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Structure Function Studies on the Fumarate Reductase of *Escherichia coli*

by

Bernard Donat Lemire

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Doctor of Philosophy

Department of Biochemistry

EDMONTON, ALBERTA

SPRING 1986

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and especially to my wife, Catherine

ABSTRACT

Fumarate reductase of *Escherichia coli* is a membrane-bound enzyme composed of a 69,000 dalton catalytic, flavin containing subunit and a 27,000 dalton non-heme iron containing subunit. Using gene cloning and amplification techniques, we have observed two additional polypeptides encoded by the *frd* operon of 15,000 and 14,000 daltons. Expression of these two small polypeptides is necessary for attachment of the two large subunits to the membrane. The holoenzyme form contains one copy of each of the four polypeptides when isolated. Unlike two subunit form, the holoenzyme is not dependent on anions for activity and is not labile at alkaline pH.

When grown anaerobically on fumarate-containing medium the membranes of the strain containing plasmid pFRD63 are highly enriched in fumarate reductase. Negatively-stained preparations of these vesicles were examined in the electron microscope where they appeared to be covered with knob-like structures about 4 nm in diameter attached to the membrane by short stalks. Treatment of the membranes with 6 M urea removed the catalytic subunits leaving the anchor polypeptides intact. Functional four subunit enzyme and the knob-like structure could be reconstituted by addition of soluble catalytic subunits to the urea-stripped membranes.

The anchor polypeptides have been analyzed for hydrophobic segments and α-helical content and a model for their organization within the lipid bilayer is presented.

In amplified membranes, greater than 50% of the inner membrane protein could be accounted for by the fumarate reductase while the total amount of protein associated with the membrane fraction doubled. The membrane accommodated this excess fumarate reductase without reducing the levels of other membrane-associated enzymes. At the same time the amount of membrane lipid increased such that the lipid protein ratio remained constant. The excess membrane was localized in novel tubular structures which were observed in thin-section and negatively-stained electron microscopic preparations. The tubules only appeared after the cytoplasmic membrane became highly enriched in fumarate reductase. They were composed of an aggregate of helically organized fumarate reductase and lipid.

We have identified a mutation within the membrane-intrinsic domain of fumarate reductase which alters the electron transfer properties of the catalytic domain. Functional electron flow from a quinone analog or from the electron transport chain is impaired. However, the mutant enzyme can be reduced normally by single electron donors such as benzyl viologen. The mutation indicates that the intrinsic and extrinsic domains are conformationally connected. These results confirm the important role of the anchor subunits in functional electron transport.

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List of Symbols and Abbreviations

$\Delta\psi$	electrical potential
ΔG	Gibb's free energy
Δp	proton motive force
ΔpH	proton gradient across the membrane
ADP	adenosine-5'-diphosphate
Amp	ampicillin
Ap	ampicillin
arg	arginine
ATP	adenosine-5'-triphosphate
BVH$^{\cdot}$	reduced benzyl viologen
CO_2	carbon dioxide
cys	cysteine
Cyt	cytochrome
DCCD	N,N'-dicyclohexylcarbodiimide
DCPIP	2,6-dichlorophenol indophenol
DEAE	diethylaminoethyl
det.	detergent
DHAP	dihydroxyacetone phosphate
DHO	dihydroorotate
DMN	2,3-dimethyl-1,4-naphthoquinone
DMSO	dimethylsulfoxide
DTSP	dithiobis (succinimidylpropionate)
DTT	dithiothreitol
E	electrochemical potential
EDTA	Ethylene diamine tetraacetate

EPR	electron paramagnetic resonance
ETC	electron transport chain
F_0	membrane domain of the ATP synthase
F_1	catalytic domain of the ATP synthase
Fe/S	non-heme iron and acid labile sulfur
Fp	Flavoprotein
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FRD	fumarate reductase
fum	fumarate
GF	glycerol-fumarate
GTP	guanosine-5'-triphosphate
G3P	glycerol-3-phosphate
G3PDH	glycerol-3-phosphate dehydrogenase
HCO_3^-	bicarbonate
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
his	histidine
HOQNO	2-n-heptyl-4-hydroxyquinoline N-oxide
ITP	inosine-5'-triphosphate
kcal	kilocalories
kD	kilodalton
Km	Michaelis constant
memb.	membrane
met	methionine
MK	menaquinone

MTT	3(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
mV	millivolt
$NaBH_4$	sodium borohydride
Na Phos	sodium phosphate
NADH/NAD+	nicotinamide adenine dinucleotide
ND	not determined
NEM	N-ethylmaleimide
NH_4OH	ammonium hydroxide
NO_2^-	nitrite
NO_3^-	nitrate
OsO_4	osmium tetroxide
ORO	orotate
P_i	inorganic phosphate
PAGE	polyacrylamide gel electrophoresis
PMS	Phenazine methosulphate
Q	ubiquinone
Quin	quinacrine
R_f	distance traveled by solute over distance traveled by solvent
sdh	succinate dehydrogenase
SDS	sodium dodecyl sulfate
Sp.Ac.	specific activity
TCA	tricarboxylic acid cycle
TEMED	tetramethylethylenediamine
tet	tetracycline

TMAO	trimethylamine N-oxide
U	units
wt	weight

I. INTRODUCTION[1]

Escherichia coli, a gram negative bacterium, is capable of deriving energy for growth both fermentatively via glycolysis, or by oxidative phosphorylation. Oxidative phosphorylation can occur aerobically with oxygen or anaerobically with nitrate, fumarate, nitrite, trimethylamine-N-oxide or dimethylsulfoxide as terminal electron acceptors (Ingledew and Poole, 1984; Bilous and Weiner, 1985). This facultative anaerobe is found in the human large intestine, an environment which provides little access to oxygen. For this reason, *E. coli* has developed a variety of enzymes which enable it to use other electron acceptors during anaerobic growth.

A. MECHANISMS OF ENERGY COUPLING

ATP synthesis can be directly or indirectly coupled to substrate oxidation to generate an energized state. Direct coupling, as exemplified by the pyruvate kinase reaction, is known as substrate level phosphorylation. This form of coupling can supply between 0 and 15% of total cellular ATP depending on the growth substrate (Holms *et al.*, 1972). Indirect coupling of substrate oxidation to ATP synthesis occurs via the respiratory chains and is known as oxidative phosphorylation. The respiratory chains generate an energized state which can in turn be used to drive energy dependent reactions.

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[1] Portions of this chapter have been published. Cole S.T., C. Condon, B.D. Lemire and J.H. Weiner. 1985. *Biochim. Biophys. Acta* 811:381-403.

Three mechanisms of energy coupling have been proposed to describe and explain this energized state. In the chemical coupling theory proposed by Slater, a high energy covalently modified electron carrier conserves redox energy. This mechanism of coupling is analogous to substrate level phosphorylation. To date, despite numerous attempts to locate one, a high energy coupling intermediate has not been described (Slater, 1953).

Consequently, Boyer suggested that the energy of redox reactions is conserved in the form of high energy protein conformations (Boyer, 1965). The energy involved in the conformational changes is transferred directly to the ATP synthase to drive the phosphorylation of ADP. This theory requires direct physical interactions between a respiratory chain protein and the ATP synthase. Reconstitution experiments where the ATP synthase has been incorporated into artificial membranes have clearly demonstrated that ATP synthesis can occur in the absence of other proteins, although conformational coupling may be important in the ATP synthase reaction itself (Kagawa and Racker, 1971; Sone *et al.*, 1977; Rögner *et al.*, 1979).

The theory which has best provided a unifying conceptual framework for energy coupling, links energy generating reactions to energy utilizing reactions by means of an electrochemical proton gradient across a proton impermeable membrane. The chemiosmotic theory was initially proposed and described by Peter Mitchell (Mitchell, 1961). It can be mathematically described as

$$\Delta p = \Delta\psi - Z\ \Delta pH$$

where Z is a constant factor of 2.303RT/F which at 25°C is approximately 60mV. Δp, the proton motive force, is the force generated by physically creating a proton gradient across a membrane. It is the sum of an electrical component, Δψ and a chemical component, ΔpH.

Four fundamental postulates describe the chemiosmotic theory (Mitchell, 1979). The first proposes the existence of a membrane-bound ATP synthase capable of reversibly interconverting the energy of the proton gradient, Δp with the chemical energy stored in the high energy molecule, ATP.

The second suggests that respiratory chains are membrane-bound entities capable of generating an electrochemical proton gradient. The respiratory components should be physically organized in the membrane in such a fashion as to vectorially translocate protons across the membrane.

Thirdly, the proton gradient generated by the respiratory chains should be capable of directly driving energy utilizing reactions such as metabolite transport by interacting with proton linked transport enzymes.

The final postulate necessary for a complete description of the theory, claims that the membrane across which the electrochemical gradient is generated must be relatively impermeable to both protons and hydroxyl ions. The fundamental principles of the chemiosmotic theory have now survived over two decades of intensive scrutiny.

A generalized scheme describing the chemiosmotic theory is seen in Figure I.1. In *E. coli*, the protons are released to the periplasmic side of the plasma membrane and the electrons are returned to the cytoplasmic side where they are used to reduce an acceptor molecule, A. The externalized protons form an electrochemical gradient which can drive the

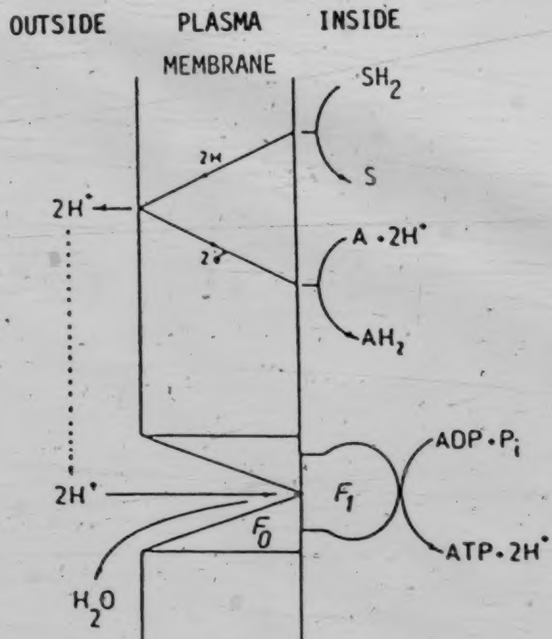

Figure I.1 Chemiosmotic respiratory loop. The substrate molecule SH_2 is enzymatically dehydrogenated and the reducing equivalents transferred to a hydrogen carrier. The protons are released at the external surface (left) of the membrane and the electrons are returned to the inner surface (right) via an electron carrier. F_1 and F_0 are domains of the ATP synthase. A=acceptor molecule. The asymmetric distribution of hydrogen and electron carriers across the membrane results in charge separation. Figure adapted from Haddock and Jones, 1977.

catalytic phosphorylation of ADP by the ATP synthase.

The chemiosmotic hypothesis makes several predictions about respiratory chains and energy coupling which have all been verified (Jones, 1982). Since ATP synthesis is solely dependent on Δp (covalent intermediates or high energy conformations are not involved) the source of the proton charge gradient should be unimportant. ATP synthesis has been coupled to Δp where Δp was generated by respiratory chains (Hinkle and McCarty, 1978), by light dependent proton pumps (Racker and Stoeckenius, 1974), or even by artificial imposition of a proton gradient (Jagendorf and Uribe, 1966).

Coupling of an electrochemical gradient has only been observed in a topologically closed system, consistent with a delocalized proton charge gradient. Oxidative phosphorylation has never been demonstrated in soluble preparations.

Further, the magnitude of the electrochemical gradient should influence the rate of respiration. This has been clearly demonstrated in both mitochondrial (Nicholls and Bernson, 1977) and bacterial systems (John and Hamilton, 1971).

Any compound which increases the permeability of protons across the membrane will decrease the magnitude of Δp (Hinkle and Horstman, 1971). Many protonophores have been shown to act as uncoupling agents decreasing or eliminating the efficiency of the conversion of the electrochemical gradient to ATP (Nicholls and Bernson, 1977).

The case of *E. coli* growing anaerobically on glycerol and fumarate provides a very simple energy conserving electron transport chain consisting of a single redox or Mitchell loop (Haddock and Jones, 1977). Glycerol crosses the cytoplasmic membrane and is phosphorylated to glycerol-3-phosphate by the enzyme glycerol kinase (Lin, 1976). Glycerol-3-phosphate serves as the reduced substrate for the anaerobic glycerol-3-phosphate dehydrogenase (Miki and Lin, 1973). Menaquinone serves as the hydrogen carrier (Newton *et al.*, 1971) and a b-type cytochrome as the electron carrier (Singh and Bragg, 1976). The electrons are used to enzymatically reduce fumarate to succinate by the membrane-bound fumarate reductase (Lin, 1976).

The oxidation of glycerol-3-phosphate coupled to fumarate reduction is capable of generating an energized membrane. This has been demonstrated by the accumulation of sugars (Rosenberg *et al.*, 1973) and amino acids in cells (Singh and Bragg, 1976) or right side out vesicles (Boonstra *et al.*, 1975; Miki and Lin, 1975), by the fumarate dependent quenching of atebrin in inside out vesicles (Haddock and Kendall-Tobias, 1975), by the fumarate dependent translocation of protons in cells (Brice *et al.*, 1974) and by the isolation of an enzyme complex which catalyzes glycerol-3-phosphate dependent fumarate reduction capable of driving ATP synthesis (Miki and Lin, 1972).

B. THE ATP SYNTHASE

The ATP synthase can be found in all energy transducing membranes such as those of the mitochondria, bacteria or chloroplasts (Hinkle and McCarty, 1978). The enzyme is a complex oligomer of at least 8 different subunits, similarly constructed in the membrane of different organisms (Futai and Kanazawa, 1983).

Seen on preparations of membranes negatively-stained with phosphotungstate, the ATP synthase appears as a globular knob approximately 100Å in diameter attached to a short stalk (Racker, 1976). The orientation of the knobs is such that they project towards the bacterial cytoplasmic space or the mitochondrial matrix. This structural orientation also corresponds to its functional orientation since ATP is hydrolysed or synthesized on the same side as the knobs (Nicholls, 1982).

The *E. coli* ATP synthase can be divided into two functional domains, a water soluble F_1 portion and a water insoluble F_0 domain. The F_1 portion corresponds to the knob and is responsible for the catalytic hydrolysis of ATP to ADP and inorganic phosphate (Downie *et al.*, 1979). This domain is composed of five subunits with a total molecular weight of about 360,000. There has been some controversy over the stoichiometry of the subunits in F_1 but it is now generally accepted to be $\alpha_3\beta_3\gamma\delta\epsilon$ (Futai and Kanazawa, 1983). The α and β subunits were thought to be capable of catalytic activity in the absence of the other subunits (Nelson *et al.*, 1974). Recently, a similar $\alpha\beta$ preparation was shown to contain proteolytic fragments of the γ subunit by immunological techniques (Smith and Sternweis, 1982). Whether parts of γ are required for activity remains to be determined.

The F_0 portion consists of three different subunits in proposed ratios of $a_1b_2c_{12-15}$ (von Meyenburg *et al.*, 1983) or $a_1b_2c_{10}$ (Foster and Fillingame, 1982). The F_0 domain has been shown to act as a proton channel across both biological (Fillingame *et al.*, 1983) and artificial (Foster and Fillingame, 1979) membranes. The F_0 is apparently a passive ion channel with a high selectivity for protons (Amzel and Pedersen, 1983). Its conducting ability is blocked by reattachment of the F_1 portion of the ATP synthase (Futai and Kanazawa, 1983).

The proteolipid or *c* subunit plays an important role in the function of F_0. Reaction with the hydrophobic compound DCCD, blocks the ability of F_0 to act as a proton channel (Rosen, 1973; Altendorf and Zitzmann, 1975). The reaction has been shown to occur with an aspartic acid residue proposed to be located in the centre of a transmembrane alpha helix (Solioz, 1984). Mutations which replace this aspartic acid residue result in a proton impermeable F_0 (Hoppe *et al.*, 1982). The isolated *c* subunit of yeast is capable of proton conduction across black lipid membranes, although at a reduced rate compared to F_0 (Schindler and Nelson, 1982).

The roles of the *a* and *b* subunits are not as well defined but both appear to be necessary for proper assembly of the proton channel and proper binding of the F_1 catalytic head (Klionsky *et al.*, 1983). A mutation in subunit *a* resulted in a DCCD insensitive ATP synthase whose F_0 showed much reduced proton conduction (Hasan *et al.*, 1978; Fillingame *et al.*, 1983). Further, the F_1 portion was more loosely attached and could be more readily dissociated from the F_0 portion. Recently, it was shown that the *a* subunit could partially collapse $\Delta\psi$ when suddenly overproduced and inserted into the membrane. This subunit may have a protonophoric activity in the ATP synthase (von Meyenburg *et al.*, 1985). Mutants in the *b* subunit resulted in high ATPase activity in the cytoplasm and a proton impermeable F_0 (Downie *et al.*, 1981).

The ATP synthase is responsible for the coupling of the electrochemical proton gradient with the chemical energy of the ATP molecule. It is capable of doing so in the absence of other proteins when reconstituted into liposomes (Sone *et al.*, 1977). Bacteria are capable of utilizing either a ΔpH or a $\Delta\psi$ to synthesize ATP (Jones, 1982).

The mechanism of ATP synthesis in the F_0F_1 ATP synthase is probably different from two other ATP hydrolysing ion pumps. In both the sodium potassium ATPase of mammalian plasma membranes and the calcium ATPase of sarcoplasmic reticulum, a phosphorylated intermediate has been detected. No such intermediate has been detected in the ATP synthase

(Sigler, 1982). Mitchell has proposed a direct model for ATP synthesis where Δp drives 2 protons through F_0. The protons attack a molecule of inorganic phosphate forming water and a reactive phosphate intermediate. This intermediate subsequently reacts with ADP to form ATP (Mitchell, 1974).

Boyer (Boyer, 1977), Slater (Slater, 1974), and others have proposed a less direct mechanism of ATP synthesis, one which emphasizes the role of the F_1 subunits. The free energy of the proton gradient causes a conformational change in F_1 which would in turn cause the synthesis of ATP by altering the relative affinities of F_1 for the reaction components. In this mechanism, the step requiring the most energy is the release of bound ATP from F_1. This model, where a conformational change is the major energy requiring step is analogous to the role of myosin in muscle contraction. Evidence that supports this latter model comes predominantly from isotope exchange studies using chloroplast or mitochondrial ATP synthase. The rate of exchange of tritium from tritiated water into chloroplast F_1 can be increased in the presence of Δp (Ryrie and Jagendorf, 1972).

That a Δp dependent change in tertiary structure occurs is also supported by altered NEM reactivity. In the absence of Δp, chloroplast F_1 will not hydrolyze ATP, but following the Δp induced conformational changes, chloroplast F_1 can be locked into an active conformation by thiol reagents or trypsin (McCarty, 1979).

Exchange of ^{18}O between water and phosphate has led Boyer to suggest that this exchange represents the equilibrium of ATP synthesis at the catalytic site in the absence of ATP release. This exchange is ADP dependent, oligomycin sensitive and Δp independent to low values of Δp, suggesting that the free energy of interconversion of ATP and ADP is not large. Similar observations on chloroplast F_1 suggest that large expenditures of energy are not required to form ATP from ADP (Feldman and Sigman, 1982). It is the release of ATP that requires large expenditures of energy (Boyer, 1977).

Boyer has proposed an alternating catalytic site mechanism for the mechanism of ATP synthesis (Boyer *et al*., 1977). An important observation is that only one Nbf-Cl (4-chloro-7-nitrobenzofuran) per enzyme is sufficient for full inhibition of activity despite the presence of three β subunits per enzyme complex (Ferguson *et al*., 1975). Further, it has been shown that bacterial F_1 contains at least two weak catalytic binding sites for ATP and ADP (Cross and Nalin, 1982). Boyer suggests that Δp causes a conformational change leading to tighter binding of ADP and phosphate at one site and to looser binding of ATP at another site. ATP is then formed at the first site while the preformed ATP at the second site is released and replaced by ADP and phosphate.

Δp is composed of an electrical component, Δψ, and of a chemical component, ΔpH. It is the magnitude of Δp rather than its composition which is of importance for driving ATP synthesis. Either component in the absence of the other can drive ATP synthesis only if the total driving force is approximately 150mV (or ΔpH is geater than 2.5 pH units) (Jones, 1982).

The primary role of the ATP synthase during fermentative growth appears to be the hydrolysis of ATP generated by substrate level phosphorylation in order to create a Δp (Harold *et al.*, 1970). Δp of magnitudes greater than 200mV have been generated in proteoliposomes suggesting that ATP hydrolysis *in vivo* is used to drive Δp dependent membrane functions (Jones, 1982).

C. THE ROLE OF THE ELECTROCHEMICAL PROTON GRADIENT

The gradient of protons across the energy-transducing membrane is capable of driving a variety of energy dependent reactions. These reactions include active transport (or the accumulation of metabolites against a concentration gradient), bacterial motility, phage infection, energy dependent transhydrogenation, reverse electron transport and of course ATP synthesis (Bragg, 1979). Figure I.2 schematically represents a general model for energy transduction.

Either component of Δp will drive ATP synthesis with a proton to phosphate ratio of two to three protons per ATP synthesized. Some types of active transport specifically require $\Delta\psi$ and/or ΔpH. Anions or neutral molecules are

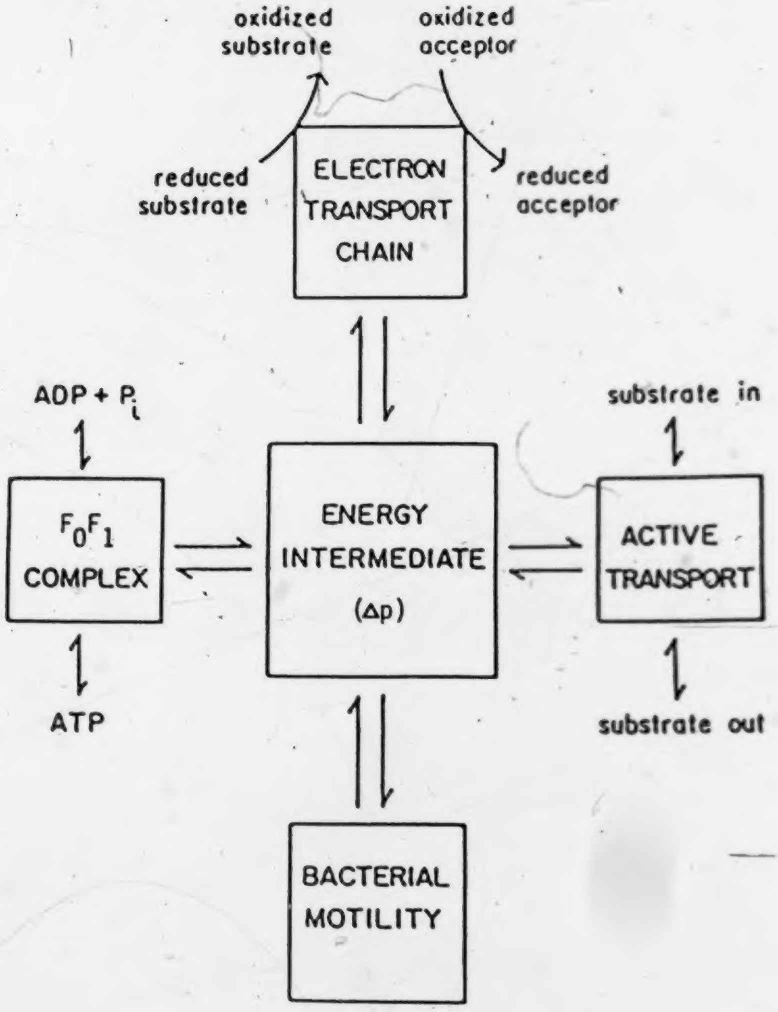
oxidized substrate
oxidized acceptor
reduced substrate
ELECTRON TRANSPORT CHAIN
reduced acceptor
ADP + P_i
substrate in
F_0F_1 COMPLEX
ENERGY INTERMEDIATE (Δp)
ACTIVE TRANSPORT
ATP
substrate out
BACTERIAL MOTILITY

Figure I.2 Energy Transduction in Membrane Systems. The energy intermediate in the centre is referred to as Δp in the text. Δp and energy requiring reactions can be readily interconverted (adapted from Robinson, 1982).

cotransported with protons (proton symport) while cations enter either with protons or unaccompanied and leave in exchange for protons (proton antiport). Examples are succinate proton symport (Gutowski and Rosenberg, 1975), sodium proton antiport (West and Mitchell, 1974) and calcium proton antiport (Tsuchiya and Rosen, 1976) as Δp dependent modes of active transport. The potassium uniport system (Harold, 1977) is Δψ dependent while the lactose proton symport (Ramos and Kaback, 1977) is Δp dependent.

D. LOCALIZED OR DELOCALIZED PROTON GRADIENTS

R.J.P. Williams has proposed an alternate model to Peter Mitchell's chemiosmotic coupling theory which states that the proton gradient is a local event and not an osmotic event (Williams, 1978b). The charge separation resulting from redox reactions is restricted to the membrane phase. Membrane-bound charges do not contribute to osmotic terms such as ΔpH or Δψ. These bound charges may not be in equilibrium with the aqueous phases. Williams criticizes Mitchell's chemiosmotic theory for its failure to account for the large buffering capacities of both external and internal aqueous phases. Very large quantities of protons would necessarily be translocated if ΔpH alone were operative in making ATP. The capacity for Δψ dependent ATP synthesis increases with larger internal volumes. It has however been experimentally observed that protons are bound by mitochondrial and thylakoid membranes. These observations cast doubt on the reliability of the values of Δψ calculated in these systems on the basis of internal volumes. Measurements of Δψ may be distorted by bound charges. These considerations lead one to ask how some bacteria are able to synthesize ATP in a medium of pH greater than 10 while others are incapable of doing so in a medium of pH less than 1.0 (Ferguson and Sorgato, 1982).

Chemiosmosis requires that the membrane separate two aqueous phases whose difference in potential meets a minimum for ATP synthesis. As suggested in the chemiosmotic theory the entire membrane must be saturated before ATP can be formed. Yet ATP synthesis can be observed following a single flash of light on the thylakoid membrane (Witt, 1975).

The localized proton gradient theory of Williams suggests that rapid movement of protons in the membrane is via a channel of bound water molecules (Williams, 1978b). Water in the region of the ATP synthase drives the reaction towards ATP synthesis. Excess energy can be liberated from the limited storage capacity of the membrane to the aqueous phase. Support for this model has come from studies with halobacteria where protons have been shown to be membrane assocciated (Michel and Oesterhelt, 1980). Further, ATP synthesis can occur in the absence of a ΔpH or even in the presence of an inverted ΔpH (Bogomolni *et al.*, 1976).

E. PROTON PUMPING REDOX COMPLEXES

Reexamination of the chemiosmotic theory's predictions of coupling ratios has been necessitated due to the measurement of $H^+/2e^-$ ratios greater than two. The respiratory loop model of Mitchell is restricted to $H^+/2e^-$ ratios of two because proton extrusion is a consequence of the transfer of electrons from a hydrogen carrier (proton plus electron) to a pure electron carrier with the release of protons (Mitchell, 1966). The electron carrier then transfers the electrons to another hydrogen carrier with the necessary uptake of protons from the medium. It is the asymmetric assembly of the chain of hydrogen and electron carriers which necessarily results in the vectorial translocation of protons.

Evaluation of oxygen pulse experiments (Brand *et al.*, 1976) suggested that the mitochondrial respiratory chain translocated more than six protons per NADH and more than four protons per succinate. These results and others have led to a conformational pump explanation. This model separates the binding sites for protons and electrons such that energy involved in redox changes is transmitted via conformational changes to a second site which responds by altering the pk_a of an ionizable group (Nicholls, 1982). The redox loop model of Mitchell also requires changes in the pk_a's of redox carriers such that oxidation and reduction events sufficiently alter the pk_a's to allow the release or uptake of protons respectively. This feature of the conformational pump model allows a structure with no redox properties to function in proton release. This may explain the overabundance of electron carriers (up to eight iron-sulfur centres, five cytochromes and two copper atoms) over hydrogen carriers (FMN and Q_{10}) in the mitochondria (Nicholls, 1982).

A second feature of conformational pumps is that there is no restriction on the stoichiometry of proton translocation per electron. The number of pk_a's affected by the conformational change induced with the reduction by a electron need not be restricted to one (Wikström and Krab, 1979).

The controversy between the redox loop and conformational pump models has been most extensively explored with mitochondrial cytochrome oxidase. Reviews of the evidence pertaining to this system, suggest cytochrome oxidase is a conformational pump and not solely an electron carrier as suggested by Mitchell (Wikström and Krab, 1979; Wikström *et al*., 1981). Very recently, Mitchell has accepted the arguments of Wikström and Casey (Wikström and Casey, 1985) and has proposed a protonmotive O loop as a possible mechanism of explaining the proton pumping of cytochrome oxidase (Mitchell *et al*., 1985).

F. THE QUINONES OF *E. coli*

E. coli can synthesize two quinones, ubiquinone-8 and menaquinone-8 (Polglase *et al*., 1965). Quinones function to carry reducing equivalents as hydrogen atoms between the dehydrogenases and the terminal oxidases or reductases. The type of quinone present is determined by the growth conditions and is linked to the amount of oxygen available

(Jones and Garland, 1982). The ratio of ubiquinone to menaquinone varies considerably from aerobically grown cells (22:1) to stationary phase cells (1.5:1) to strictly anaerobically grown cells where ubiquinone is undetectable (Polglase *et al.*, 1965). The coupling of various dehydrogenases to the terminal enzyme complexes shows some specificity for the type of quinone used. Ubiquinone is able to reconstitute the oxidase activities of NADH, D-lactate, succinate and glycerol-3-phosphate dehydrogenases when added exogenously to membranes of an aerobically grown double quinone mutant (Wallace and Young, 1977). Menaquinone was able to function fully with glycerol-3-phosphate dehydrogenase and partly with D-lactate dehydrogenase but not with NADH or succinate dehydrogenases. There is still controversy however over the location of ubiquinone within the aerobic electron transport chain (Au *et al.*, 1984).

G. AEROBIC ELECTRON TRANSPORT CHAIN

Investigation of the membrane-bound cytochromes of aerobically grown E. coli has revealed the presence of seven cytochromes which include five b-type cytochromes, cytochrome a_1 and cytochrome d (Scott and Poole, 1982). There appear to be two terminal branches leading to oxygen, the presence and levels of the individual components being determined by the oxygen tension during growth. At least two cytochromes are capable of functioning as terminal oxidases; cytochrome o and cytochrome d (Ingledew and

Poole, 1984). The aerobic electron transport chain probably consists only of b-type cytochromes, as electron transport is restored with hematin and ATP in membranes of heme synthesis deficient mutants (Haddock, 1973).

Cells grown under conditions of high aeration contain two cytochromes; cytochrome b_{556} and cytochrome o (Lorence, *et al.*, 1984). However, both of these cytochromes are present under a variety of growth conditions and are sometimes considered to be constitutively synthesized (Haddock and Jones, 1977). Cytochrome o is a b-type cytochrome which can bind carbon monoxide and cyanide and functions as a cytochrome oxidase. It has been proposed that cytochrome o has a split α band with absorbance maxima at 555 and 562 nm. Potentiometric titrations in the presence and absence of carbon monoxide suggest that cytochrome b_{555} and cytochrome b_{562} represent a single cytochrome identical to cytochrome o (Lorence, *et al.*, 1984). Purification of a cytochrome b_{562} cytochrome o complex resulted in a preparation showing two bands of 55,000 and 33,000 daltons as determined by SDS PAGE (Kita *et al.*, 1984a). The bands are present in equimolar amounts. The preparation contains heme and copper but no detectable non-heme iron, quinone or phospholipid. Difference spectra at 77°K show two α peaks at 555 and 562 nm. The cytochrome o-b_{562} segment of the aerobic chain is capable of generating a membrane potential in the presence of ubiquinol analogs using ascorbate and PMS as electron donors (Kita *et al.*, 1982). Reconstitution of a different cytochrome o preparation with the lac carrier protein could cause the accumulation of lactose with ubiquinol as electron donor (Matsushita *et al.*, 1983).

The product of the oxygen reduction reaction has not been firmly established. Rate and stoichiometry suggest that four electrons from NADH can be used to reduce oxygen to water (Ingledew and Poole, 1984). However, reduction of oxygen in liposomes is enhanced by adding superoxide dismutase, suggesting that superoxide anions are products of either the ubiquinone 1 oxidation or of oxygen reduction (Matsushita *et al.*, 1983).

Cytochrome d functions as an alternative oxidase in *E. coli* (Green and Gennis, 1983). Its synthesis seems to be coordinated with cytochrome a_1 and cytochrome b_{558} (Ingledew and Poole, 1984) and is favored under conditions of lowered oxygen tension (Rice and Hempfling, 1978), stationary phase growth (Sweet and Peterson, 1978), and anaerobic growth (Haddock *et al.*, 1976). Growth in the presence of cyanide (Ashcroft and Haddock, 1975) or under sulphate limiting conditions also enhances synthesis (Poole and Haddock, 1975).

Cytochrome d appears to be less efficient at oxidative phosphorylation but its higher affinity for oxygen offers it a competitive advantage under conditions of oxygen limitation (Rice and Hempfling, 1978). A purified preparation of cytochrome b_{556} cytochrome d complex isolated from *E. coli* grown under oxygen limiting conditions showed two bands of protein at 26,000 and 51,000 daltons after SDS PAGE (Kita *et al.*, 1984b). Both proteins were present in equimolar amounts and contained heme and chlorin heme typical of cytochrome d. Spectrally the complex absorbed at 558 and 624nm. A similar purified preparation which contained cytochrome d, a_1 and b_{558} showed bands at 57,000 and 43,000 daltons after SDS PAGE. The complex has absorption maxima at 558.5 and 624.5nm (Miller and Gennis, 1983).

The complex showed a higher affinity for oxygen than the purified cytochrome o. Upon reconstitution, cytochrome d is able to generate a membrane potential with ubiquinol donors (Koland *et al.*, 1984; Kita *et al.*, 1984b). Corrected proton to oxygen ratios of 1.7 were obtained with reconstituted cytochrome d oxidizing quinones. Some authors suggest that these ratios can be explained solely by the scalar protons involved in the reaction (Miller and Gennis, 1985).

The role of cytochrome a_1 in *E. coli* is obscure. Difficulties in assigning its absorption spectrum have created confusion. With one exception, cytochrome a_1 has failed to demonstrate an oxidase function (Ingledew and Poole, 1984). Cytochrome a_1 appears to be coordinately synthesized with cytochrome d and appears to be able to bind carbon monoxide and function as a terminal oxidase (Poole *et al.*, 1981; Edwards *et al.*, 1981). Other investigators suggest cytochrome a_1 may exist as a complex with cytochrome d (Miller and Gennis, 1983).

Aerobically grown cells contain two to four b-type cytochromes as distinguished by potentiometric and spectrophotometric properties (Ingledew and Poole, 1984). Their orders and functions in the electron transport chain remain to be clearly demonstrated.

Using a ubiquinone deficient mutant, Downie and Cox (1978) have presented evidence suggesting quinones function at two locations. This scheme is not supported by the majority of published midpoint potentials (Ingledew and Poole, 1984). It is also in conflict with the copurification of a cytochrome b_{562}-cytochrome o complex (Kita *et al.*, 1984a).

A similar approach has resulted in a different scheme for the electron transport chain as shown in Figure I.3 (Kita and Anraku, 1981). Support for this model comes from

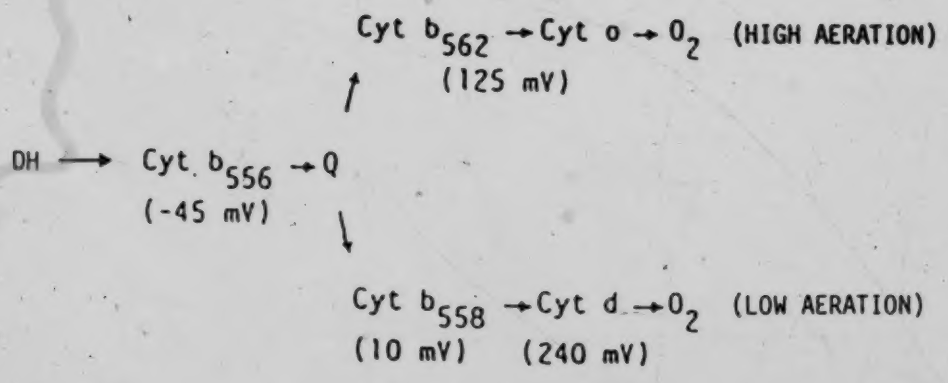
Cyt b_{562} → Cyt o → O_2 (HIGH AERATION)
(125 mV)
DH → Cyt b_{556} → Q
(-45 mV)
Cyt b_{558} → Cyt d → O_2 (LOW AERATION)
(10 mV)
(240 mV)

Figure I.3 Arrangement of cytochromes in the aerobic respiratory chain. Arrangement of cytochromes in the respiratory chain of cells of *E. coli* in late exponential phase of aerobic growth (adapted from Kita *et al.*, 1984b). Q=ubiquinone-8; cyt=cytochrome; DH=dehydrogenase.

the copurifications of cytochrome b_{562} with cytochrome o and cytochrome b_{558} with cytochrome d, suggesting associations in the respiratory chain. The authors later suggested that the branch point to cytochrome d occurs at ubiquinone, based on steady state kinetic measurements (Kita *et al*., 1984b).

THE COUPLING SITES OF THE AEROBIC CHAIN

Measurements of the stoichiometry of respiration driven proton translocation in aerobically grown cells suggest that the aerobic electron transport chain consists of two energy conserving sites (Brice *et al*., 1974). Proton to oxygen ratios of four for malate oxidation and two for succinate oxidation suggest that one coupling site is associated with the NADH dehydrogenase segment of the chain while the other is between the quinone and cytochrome o (Lawford and Haddock, 1973). Molar growth yields determined in continuous culture suggest four moles of ATP are made per mole of oxygen consumed (Farmer and Jones, 1976).

Under certain conditions, the coupling site associated with NADH dehydrogenase can be lost resulting in proton to oxygen ratios of two for malate oxidation (Poole and Haddock, 1975). Purification and reconstitution of complexes containing either cytochrome o or cytochrome d has supported the placement of an energy coupling site in these segments of the chain (Koland *et al*., 1984; Kita *et al*., 1984a).

H. ANAEROBIC ELECTRON TRANSPORT CHAINS AND THEIR TERMINAL REDUCTASES

ELECTRON TRANSPORT CHAIN TO FUMARATE

When *E. coli* is grown anaerobically in the presence of fumarate and the absence of nitrate, a membrane-bound fumarate reductase is induced. Fumarate serves as a terminal electron acceptor allowing the reoxidation of NADH (Ingledew and Poole, 1984). Fumarate reductase and its associated electron transport chain can couple to such substrates as glycerol-3-phosphate, formate, NADH, lactate, hydrogen and dihydroorotate (Ingledew and Poole, 1984).

Fumarate reductase faces the cytoplasm as depicted in Figure I.4 (Haddock and Jones, 1977). The location of the active site of fumarate reductase was determined using membrane impermeable redox dyes (Jones and Garland, 1977), immunoelectrophoresis and immunoabsorption techniques (van der Plas *et al.*, 1983) and by radioiodination of spheroplasts and everted membrane vesicles (Robinson, 1982).

Respiration driven proton translocation has been demonstrated in anaerobic cell suspensions pulsed with fumarate (Brice *et al.*, 1974). The number of protons ejected per fumarate was measured as 1.15H^+/fumarate. When glycerol-3-phosphate is used as substrate , ratios of approximately two protons per dihydroxyacetonephosphate are obtained (Miki and Lin, 1975). Gutowski and Rosenberg (1977) have shown that fumarate enters the cells by proton symport thereby causing

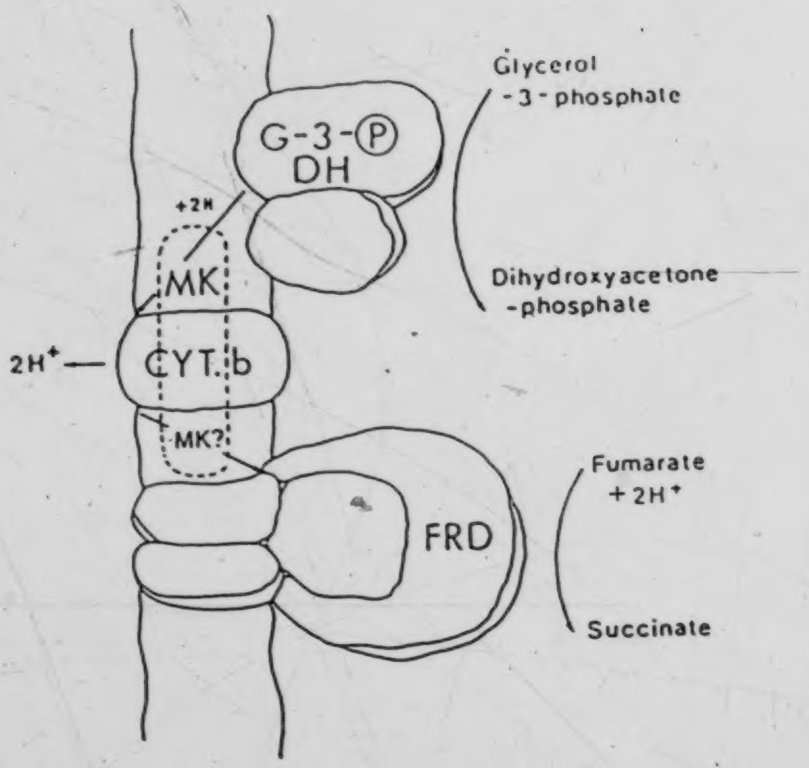

Figure I.4 Anaerobic electron transport chain to fumarate. The proposed electron transport chain from glycerol-3-phosphate to fumarate transfers reducing equivalents from the primary dehydrogenase (G-3-PDH) to the lipid soluble hydrogen carrier menaquinone (MK) (Cole *et al*., 1985). The electrons are transferred to the b-type cytochrome, protons extruded and electrons transferred to fumarate reductase (FRD). CYT=cytochrome. Both the dehydrogenase and the reductase catalyze their respective reactions on the cytoplasmic side of the membrane.

an underestimation of the number of protons translocated.

The oxidation of glycerol-3-phosphate coupled to fumarate reduction is capable of generating an energized membrane as demonstrated by the accumulation of sugars (Rosenberg *et al*., 1975) or amino acids (Singh and Bragg, 1976) in cells and right side out vesicles (Boonstra *et al*., 1975;

Miki and Lin, 1975), by fumarate dependent quenching of atebrin in inside out vesicles (Haddock and Kendall-Tobias, 1975), and by the isolation of an enzyme complex which catalyzes glycerol-3-phosphate dependent fumarate reduction capable of driving ATP synthesis (Miki and Lin, 1972).

The use of mutants has demonstrated that menaquinone is essential for fumarate reduction (Newton *et al.*, 1971). A heme deficient mutant was able to grow anaerobically on glycerol and fumarate (Singh and Bragg, 1975) but was unable to support fumarate dependent active transport (Singh and Bragg, 1976). The absence of a functional cytochrome did not inhibit electron transport to fumarate sufficiently to prevent the reoxidation of NADH and therefore allowed glycolytic growth. It did however prevent a fumarate dependent vectorial translocation of protons (Haddock and Jones, 1977).

FUMARATE REDUCTASE

The fumarate reductase of *E. coli* is a membrane-bound flavoprotein induced during anaerobic growth on fumarate (Haddock and Jones, 1977). The enzyme contains four nonidentical subunits present in equimolar amounts. The largest subunit of 69,000 daltons contains a covalently bound flavin adenine dinucleotide cofactor and may contain an iron-sulfur cluster of the [4Fe-4S] type (Weiner and Dickie, 1979; Ingledew and Poole, 1984; Johnson *et al*., 1985b). The 27,000 dalton subunit has, on the basis of the arrangement of cysteine residues in its primary sequence, been deduced to contain two or three iron-sulfur clusters (Cole *et al*., 1985). Recent electron paramagnetic resonance and magnetic circular dichroism studies (Morningstar *et al*., 1985; Johnson *et al*., 1985a) have confirmed the presence of one [2Fe-2S] and one [3Fe-xS] centre in fumarate reductase. The [3Fe-xS] centre (centre 3) was apparently more labile in the solubilized enzyme. Together the 69,000 and 27,000 dalton subunits form a catalytically active dimer capable of reducing fumarate with artificial donors. This form of the enzyme has been purified and well characterized (Dickie and Weiner, 1979; Robinson and Weiner, 1982). An important property of the dimeric enzyme, one that has been used to distinguish it from the native tetrameric form, is its dependence on anions for maximal activity and stability (Robinson and Weiner, 1981; Lemire *et al*., 1982).

The fumarate reductase operon has been cloned and sequenced (Cole, 1982; Cole *et al*., 1982; Grundström and Jaurin, 1982). In addition to the genes coding for the 69,000 and 27,000 dalton subunits, two genes coding for polypeptides of 15,000 and 13,000 daltons are present. These polypeptides have predicted amino acid sequences which are extremely hydrophobic and have been shown to function as anchors for the catalytic dimer holding it to the cytoplasmic face of the membrane. In addition, their presence modifies the catalytic and stability properties of the dimeric enzyme, removing its dependence on anions (Lemire, *et al.*, 1982).

Fumarate reductase faces the cytoplasm as depicted in Figure I.4. The organization of the tetrameric holoenzyme in the membrane is such that the anchor subunits, which are potentially transmembranal, hold the dimer on the inner face of the cytoplasmic membrane as seen in Figure I.5 (Lemire *et al.*, 1983). This has been shown by the use of permeable and nonpermeable redox dyes to donate reducing equivalents to either intact cells or everted membrane vesicles (Weiner *et al.*, 1984b). Further, immunoabsorption techniques localize the catalytic subunits to the cytoplasmic side of the membrane (van der Plas *et al.*, 1983).

The roles of the C and D subunits in fumarate reductase have recently been proposed to include interaction with the quinone component of the electron transport chain (Weiner *et al.*, 1986; Cecchini *et al.*, 1984b). Neither peptide C or D by itself is capable of supporting quinone reduction or membrane binding (Cecchini *et al.*, 1984a).

ENERGETICS OF THE GLYCEROL TO FUMARATE CHAIN

The free energy derived from the oxidation of glycerol-3-phosphate coupled to the reduction of fumarate is sufficient to translocate 2 protons per molecule of glycerol-3-phosphate oxidized. The standard redox potentials of the glycerol-3-phosphate to dihydroxyacetonephosphate and of the

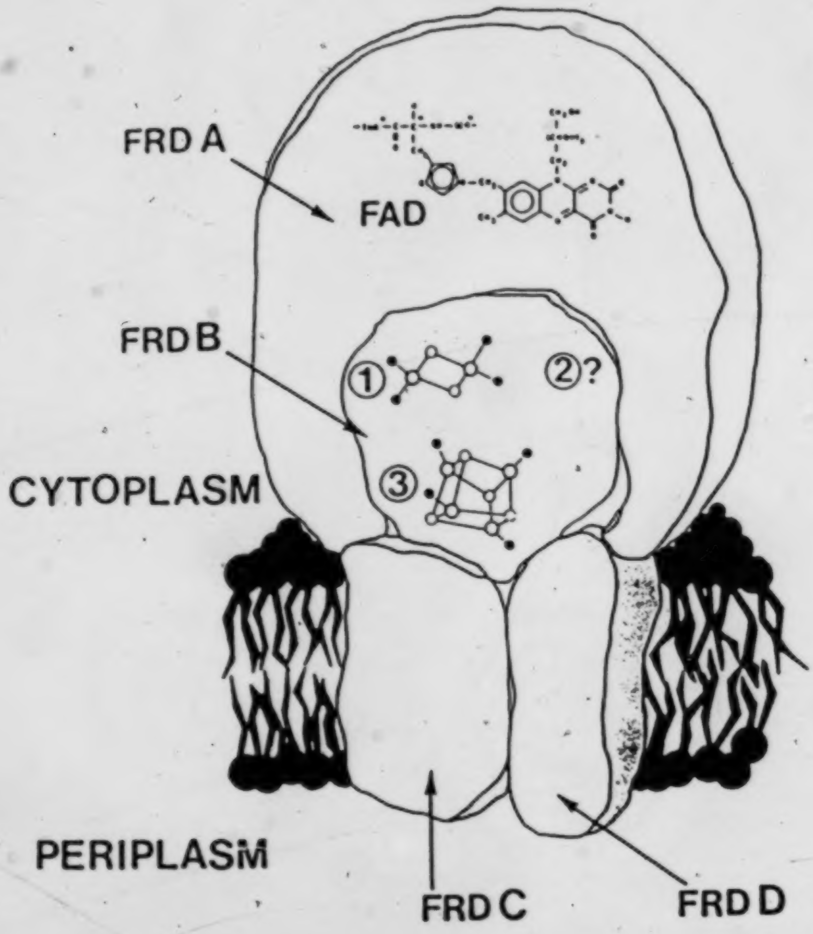

Figure I.5 A model for the arrangement of fumarate reductase in the membrane. The proposed locations of the redox centres (iron-sulfur centres 1, 2 and 3 as well as the covalent flavin cofactor) are shown (Cole *et al.*, 1985). Centre 2 is likely an [4Fe-4S] cluster while centre 3 is likely an [3Fe-xS] cluster (Johnson *et al.*, 1985a; Johnson *et al.*, 1985b).

fumarate to succinate couples are -190mV and +30mV respectively as seen in Table I.1 (Kröger, 1977). The difference in potential of these couples is 220mV which corresponds to a $\Delta G^{\circ\prime}$ of 10.0 kcal per pair of electrons. The relationship between electrochemical potential and free energy can be

Table I.1

Standard redox potentials and free energy changes

Redox Couples	E'_o (mV)	ΔG with fum (kcal/mole)	P/fum maximum
$H_2/2H^+$	-420	-20.6	1.7
Formate/HCO_3^-	-416	-20.4	1.7
NADH/NAD^+	-320	-16.0	1.3
lactate/pyruvate	-197	-10.3	0.86
G3P/DHAP	-190	-10.0	0.83
Malate/oxaloacetate	-172	- 9.2	0.83
Fumarate/succinate	+ 30	--	--

standard redox potentials=E'_o; ΔG=free energy derived from coupling to fumarate/succinate redox couple; P/fum=number of high energy phosphate bonds which could be produced when oxidation is coupled to fumarate reduction; G3P=glycerol-3-phosphate DHAP=dihydroxyacetone phosphate

expressed as

$$\Delta G^\circ = -nFE^\circ$$

where n is the number of electrons involved, F=23000 calories per mV and E is the standard potential. The calculated free energy of 10.0 kcal per pair of electrons assumes standard potentials. The situation existing in the bacterial cell is likely to deviate from standard conditions since these assume equimolar concentrations of donor and acceptor molecules involved in the redox reaction. The free energy of coupling glycerol-3-phosphate oxidation to fumarate reduction may be substantially different from 10.0 kcal.

The energy required to phosphorylate ADP to ATP in mitochondria is about 12 kcal per mole (Guynn and Veech, 1973). However, there is some controversy as to the stoichiometry of protons necessary to drive the phosphorylation of ADP. The number of protons required to phosphorylate ADP to ATP is apparently different in mitochondria, chloroplasts and bacteria. Furthermore, the phosphorylation potential may be closer to about 11 kcal per mole in bacteria (Ferguson and Sorgato, 1982). The uncertainty regarding the energetic parameters of various systems renders it impossible to ascertain the true efficiency of this particular chain.

Kinetic measurements performed on submitochondrial particles have suggested an H^+/ATP ratio of 2 (Thayer and Hinkle, 1973). However, thermodynamic measurements of the H^+/ATP ratio done on brown adipose tissue mitochondria give values approaching 3 (Nicholls and Bernson, 1977). More recent kinetic measurements of the number of protons translocated upon hydrolysis of ATP give values of three (Alexandre *et al.*, 1978). Measurements of the H^+/O ratio for succinate oxidation in rat liver mitochondria give values of 8 (Costa *et al.*, 1984); this is consistent with an H^+/ATP ratio of 3 (Cross, 1981). The H^+/ATP ratio in chloroplasts has also been measured and the values range from 2.5 to 3.1 (McCarty, 1978; Williams, 1978). Measurements of the number of protons ejected per site also suggest a value of three (Junge *et al.*, 1970).

The situation in bacteria is also unclear. Thermodynamic measurements of H^+/ATP ratios in whole cells, inside out vesicles or chromatophores from organisms such as *P. denitrificans*, *R.capsulata*, *T thermophilus* and

M. methylotrophus range from 2 to 3. Kinetic measurements of ATP synthesis in whole cells of *Streptococcus lactis* subjected to an artificially generated Δp yield H^+/ATP ratios of 1.8 (Jones, 1982). However, the H^+/ATP ratios need not be identical in all systems. H^+/ATP ratios of 2 or 3 would suggest either 0.66 or 1 ATP are synthesized by oxidative phosphorylation per glycerol-3-phosphate oxidized respectively. Growth of *E.coli* anaerobically on fumarate with either formate or hydrogen as energy sources are in agreement with a maximum value of 1 ATP per fumarate reduced (Bernhard and Gottschalk, 1978).

ELECTRON TRANSPORT CHAIN TO NITRATE

When grown under anaerobic conditions in the presence of nitrate and molybdenum, *E. coli* synthesizes a membrane-bound electron transport chain that allows growth on such non-fermentable carbon sources as D-lactate (Showe and De Moss, 1968). Two membrane-bound components, cytochrome b_{556} and nitrate reductase, are synthesized in the absence of oxygen.

If potassium selenite is also included in the growth medium high levels of membrane-bound formate dehydrogenase are also induced. Suggestions have been made that formate dehydrogenase is the normal donor of reducing equivalents to nitrate reductase (Ruiz-Herrera and De Moss, 1969). However, other physiological reductants such as NADH, glycerol-3-phosphate and D-lactate also interact via their respective dehydrogenases (Haddock and Jones, 1977).

The organization of the components in the membrane is seen in Figure I.6 (Haddock and Jones, 1977). Evidence that the site of NO_3 reduction faces the periplasmic space has

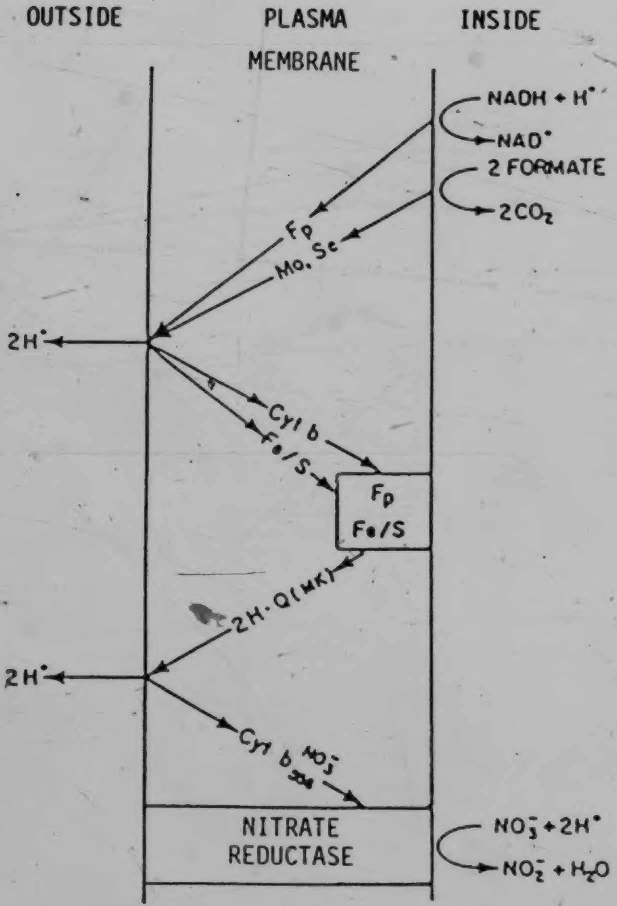
OUTSIDE
PLASMA MEMBRANE
INSIDE
NADH + H+
NAD+
2 FORMATE
2CO2
Fp
Mo, Se
2H+
Cyt b
Fe/S
Fp
Fe/S
2H·Q(MK)
2H+
Cyt b
NO3-
NITRATE REDUCTASE
NO3- + 2H+
NO2- + H2O

Figure I.6 Anaerobic electron transport chain to nitrate. The anaerobic electron transport chain to nitrate reductase arranged across the *E. coli* membrane (adapted from Haddock and Jones, 1977). Mo,Se is the molybdenum and selenium containing formate dehydrogenase. Mk=menaquinone; Fp=flavoprotein; Fe/S=iron-sulfur protein(s); Q=ubiquinone; cyt=cytochrome.

come from experiments estimating the permeability of the membrane to NO_3^-. The measured permeability rate failed to accomodate the observed activities of nitrate reductase (Garland *et al.*, 1975). The results of such measurements cannot account for the presence of a low Km nitrate transporter or of a nitrate-nitrite antiporter. More recent data using azide ion, a competitive inhibitor of nitrate reductase, have suggested the site of nitrate reduction may be on the cytoplasmic face (Jones *et al.*, 1978). Also, studies with chlorate, an analog of nitrate reduced by nitrate reductase, support the localization of the active site on the cytoplasmic side of the membrane. These results strongly suggest the presence of an as yet unidentified nitrate-nitrite antiport system (Ingledew and Poole, 1984).

The nitrate associated electron transport chain is capable of proton translocation. Stoichiometries of approximately 4 protons per nitrate have been determined when coupling is to NADH or formate (Brice *et al.*, 1974). Succinate, D-lactate or glycerol result in stoichiometries of two protons per nitrate (Garland *et al.*, 1975). That the nitrate reductase associated electron transport chain generates a Δp has been shown by atebrin fluorescence quenching in inverted membrane vesicles (Haddock and Kendall-Tobias, 1975) and by the distribution of lipophilic cations and weak acids (Boonstra and Konings, 1977) in response to an electrochemical potential.

The nature of the quinone associated with nitrate reductase is unclear. Both ubiquinone and menaquinone are synthesized during anaerobic growth on nitrate. Ubiquinone is more efficient in reconstitution experiments (Enoch and Lester, 1974) but ubiquinone deficient mutants grow equally well as the parent strain (Cox *et al.*, 1970).

NITRATE REDUCTASE

Nitrate reductase is an intrinsic membrane enzyme containing molybdenum, non-heme iron and acid-labile sulfur (Haddock and Jones, 1977). The enzyme is a complex of three nonidentical subunits α, β and γ with a complex native molecular weight of about 500,000 . The holoenzyme is composed of two α (150,000 daltons each), two β (60,000 daltons each) and four γ (20,000 daltons each) polypeptides (Ingledew and Poole, 1984). The γ subunit, referred to as cytochrome b_{556}, subunit is a b-type cytochrome whose presence is required for quinol oxidation but not for oxidation of redox dyes (MacGregor, 1975). The catalytic activity apparently resides in the α subunit (Ingledew and Poole, 1984). The role of the β subunit is unclear. This subunit exists in two forms, β and β' with apparent molecular weights of 60,000 and 58,000 respectively as determined by SDS-PAGE (Ingledew and Poole, 1984). These forms are not related by proteolysis and can be reversibly interconverted (Chaudry *et al.*, 1983), possibly by addition or removal with carbohydrate (Forget and Rimassa, 1977). The presence of γ

subunit is essential for membrane attachment (Azoulay *et al.*, 1977).

Nitrate reductase has been shown to be transmembranal (Boxer and Clegg, 1975) with the γ subunit being exposed to the periplasmic membrane surface. The α and β subunits appear to be exclusively exposed on the cytoplasmic membrane surface (Ingledew and Poole, 1984).

Many nitrate reductase mutants have been identified by selection for growth anaerobically in the presence of chlorate (Ruiz-Herrera *et al.*, 1969). A functional nitrate reductase is able to reduce chlorate to the toxic chlorite. The genes *chl*A, *chl*B, *chl*D, *chl*E and *chl*G are involved in transport and processing of molybdenum necessary for proper insertion of the cofactor into either nitrate reductase or formate dehydrogenase (Ingledew and Poole, 1984). The nitrate reductase operon contains the genes coding for the α, β and the γ subunits, *nar*G, H, and I respectively. In addition, the *nar*L locus maps close but outside the nitrate reductase operon and is apparently involved in induction of enzyme activity in response to nitrate (Li *et al.*, 1985; Stewart, 1982).

ELECTRON TRANSPORT CHAIN TO NITRITE

In the absence of oxygen, *E. coli* can reduce nitrite to ammonia. Three separate pathways have been shown to exist, a soluble NADPH:sulfite reductase also capable of reducing nitrite, a soluble NADH:nitrite reductase and a membrane associated nitrite reductase (Ingledew and Poole, 1984).

Membrane associated nitrite reduction has been linked to the oxidation of lactate or formate (Ingledew and Poole, 1984; Pope and Cole, 1982). Very little is known of the electron transport chain because this activity is lost upon cell lysis (Abou-Jaoudé *et al.*, 1979a). It is ubiquinone dependent (Abou-Jaoudé *et al.*, 1979c), and cytochrome c_{552} appears to participate in the chain since nitrite reduction does not occur in heme deficient mutants (Abou-Jaoudé *et al.*, 1979b). Purified cytochrome c_{552} has been shown to reduce nitrite (Liu *et al.*, 1981) and to be located in the periplasmic space (Cole, 1968). Formate dependent nitrite reduction is capable of generating a $\Delta\psi$ (Motteram *et al.*, 1981).

NITRITE REDUCTASE

Nitrite reductase activity is believed to be associated with cytochrome c_{552} whose absence in a *nir*197 strain correlates with the absence of membrane associated nitrite reductase activity (Abou-Jaoudé *et al.*, 1979b). Furthermore, the presence of nitrite induces the synthesis of cytochrome c_{552}.

Cytochrome c_{552}, a polypeptide localized in the periplasmic gel, contains four to six hemes and is rapidly oxidized by nitrite. It has a midpoint potential of -194 to -220mV and when purified is capable of reducing nitrite to ammonia using artificial electron donors (Liu *et al.*, 1981).

The activity is sensitive to nitrate and copper but not to azide. The presence of a *chlI* mutation (cytochrome b_{556} which is associated with nitrate reductase) has no effect on nitrite reduction.

The sensitivity of the nitrite reductase activity to cell disruption, combined with the presence of at least two other such activities has made the study of this membrane linked enzyme difficult.

ELECTRON TRANSPORT CHAIN TO TRIMETHYLAMINE-N-OXIDE

Trimethylamine-N-oxide (TMAO), a constituent of fish and invertebrates, can be reduced to trimethylamine by *E. coli* (Tarr, 1954). TMAO allows the anaerobic growth of *E. coli* on formate or hydrogen and stimulates anaerobic growth on glucose (Ingledew and Poole, 1984). Proton extrusion and the generation of a membrane potential coupled to TMAO reduction has been observed in anaerobically grown *E. coli* (Takagi *et al.*, 1981).

Shimokawa and Ishimoto (1979) have postulated the existence of four trimethylamine N-oxide reductases in *E. coli*. One of these is constitutively expressed while the other three are substrate inducible. Pascal *et al.* (1984) showed that the major inducible enzyme accounts for 93% of the total TMAO reductase activity and is coded for by the *tor*A gene. This major form is membrane associated and has been purified to near homogeneity (Shimokawa and Ishimoto, 1979). A second inducible enzyme accounted for 4%

of the total activity while a third activity was constitutive and accounted for only 2.5% of the total activity (Pascal *et al.*, 1984). These authors did not report the presence of a fourth activity.

Anaerobic growth on TMAO induces the formation of a new cytochrome pathway capable of transferring electrons from formate and NADH. These cytochromes have α absorption bands at 548, 552, 554 and 557nm and can be rapidly oxidized by TMAO. Copper sulfate inhibited the oxidation of the first two cytochromes while HOQNO had no effect. Bragg and Hackett (1983) have proposed an electron transport chain of the following order: substrates to cytochromes 548,552 to cytochromes 554,557 to TMAO reductase to TMAO. Experiments using the membrane impermeant oxidant, ammonium persulfate, led the authors to suggest that some of the components of this chain are localized in the periplasmic space.

Anaerobic transport to TMAO is quinone and cytochrome dependent (Cox and Knight, 1981). It was recently shown that menaquinone was specifically required for TMAO reduction in *E. coli* (Meganathan, 1984).

TMAO REDUCTASE .

The major TMAO reductase of *E. coli* is the terminal anaerobic electron transport enzyme to trimethylamine-N-oxide and is distinct from the constitutively synthesized enzyme (Violet *et al.*, 1985). It is a membrane-bound enzyme coded for by the *tor*A gene (Pascal *et al*., 1984). The extent of association with the membrane apparently varies with the method of cell disruption used (Violet *et al*., 1985). The enzyme has been purified to near homogeneity and was capable of reducing N-oxides of purines and pyrimidines as well as TMAO, hydroxylamine, chlorate and bromate. The enzyme is capable of accepting reducing equivalents from FMN, FAD and benzyl or methyl viologens but not from cytochrome c. It is activated by ferric ion and inhibited by cupric ions. The native molecular weight as determined by gel filtration was 220,000. Two non-identical subunits of 60,000 and 80,000 daltons are present following electrophoresis in the presence of SDS (Shimokawa and Ishimoto, 1979).

TMAO reductase contains 1.8 moles of iron per mole of enzyme and requires a molybdenum cofactor (Shimokawa and Ishimoto, 1979; Cox and Knight, 1981). TMAO reductase activity is unaffected by *nar* G, H or I mutations, indicating it does not share any polypeptides with nitrate reductase (Takagi *et al*., 1981). However, a *chl*D mutation affects both the TMAO and the nitrate reductases indicating a molybdenum cofactor is present in both enzymes (Cox and Knight, 1981).

Using *tor-lac* fusions, Pascal *et al*. (1984) showed that the expression of the *tor*A gene is not affected by the presence of nitrate or a *nir*R (*fnr*) background. However, both the presence of nitrate or an *fnr* mutation reduce the levels of measured TMAO reductase activity. These effects were attributed to something other than the expression of the *torA* gene.

ELECTRON TRANSPORT CHAIN TO DIMETHYLSULFOXIDE

Dimethylsulfoxide (DMSO) allows the anaerobic growth of *E. coli* on glycerol with the production of dimethylsulfide. The enzyme, DMSO reductase, appears to be different from the other known reductases (Bilous and Weiner, 1985a). It requires molybdenum for activity, appears to be preferentially membrane associated and is capable of translocating protons in whole cells with a ratio of four protons per DMSO. The enzyme is induced under anaerobic conditions in the absence of nitrate. Proton translocation is HOQNO sensitive suggesting the presence of a b-type cytochrome (Bilous and Weiner, 1985b).

DMSO REDUCTASE

DMSO reductase is a membrane associated activity repressed by aerobic growth or anaerobic growth on nitrate. It is under the control of the *fnr* gene product (Bilous and Weiner, 1985a). It is unlikely that this activity is associated with the biotin sulfoxide reductase since this latter enzyme is soluble and synthesized constitutively (Del-Campillo-Campbell and Campbell, 1982). Furthermore, a *bisC* mutant had no effect on anaerobic growth with DMSO as electron acceptor (Bilous and Weiner, 1985a).

Interestingly, methionine sulfoxide can replace DMSO as terminal electron acceptor (Bilous and Weiner, 1985a). A soluble methionine sulfoxide reductase activity has been purified although a significant fraction of the activity was membrane associated (Ejiri *et al.*, 1980). Whether the methionine sulfoxide reductase activity requires a molybdenum cofactor is unknown. Bilous and Weiner (1985a) having shown molybdenum is required for the DMSO reductase activity, suggested that a single distinct enzyme may be responsible for the reduction of both DMSO and methionine sulfoxide.

Several similarities between TMAO and DMSO reductases exist. Both require a molybdenum cofactor, both are repressed by aerobic growth or anaerobic growth on nitrate and both are sensitive to exogenous tungstate (Bilous and Weiner, 1985a). The relatedness of these enzymes is obscured by the presence of three or four enzymes capable of reducing TMAO (Pascal *et al.*, 1984). Purified TMAO reductase was unable to reduce methionine sulfoxide; however, only one of the three TMAO reductases was examined (Sagai and Ishimoto, 1973). Styrvold and Strom (1984) could not separate the DMSO and TMAO reductase activities of *P. Vulgaris* or *E. coli* by chromatographic or electrophoretic procedures. Both activities seemed to have the same molecular weights and isoelectric points.

I. PRIMARY DEHYDROGENASES

GLYCEROL-3-PHOSPHATE DEHYDROGENASES

E. coli can grow with glycerol as sole carbon and energy source in the presence of oxygen, nitrate, fumarate or other terminal electron acceptors (Haddock and Jones, 1977; Bilous and Weiner, 1985a). Two different membrane associated dehydrogenases have been identified and these are termed aerobic and anaerobic because of their differing interactions with the aerobic electron transport chain. Using an *E. coli* mutant deficient in the aerobic glycerol-3-phosphate dehydrogenase, encoded by the *glp*D gene, Kistler and Lin found that anaerobic growth on fumarate or nitrate was normal but halted upon the admission of oxygen (Kistler and Lin, 1972a). Mutants lacking the anaerobic enzyme, encoded by the *glp*AB genes, could grow to normal levels aerobically or anaerobically on nitrate but not on fumarate. Together these results suggest that the aerobic enzyme is present under both anaerobic and aerobic conditions. It can couple with the electron transport chain to nitrate or oxygen but not that leading to fumarate. The anaerobic enzyme is apparently unable to couple with the aerobic electron transport chain but can couple to nitrate or fumarate. Its levels were found to be elevated several fold when fumarate was the electron acceptor instead of nitrate or oxygen.

The aerobic sn-glycerol-3-phosphate dehydrogenase is an abundant membrane-bound respiratory enzyme which can comprise up to 10% of the inner membrane protein in constitutive strains (Weiner and Heppel, 1972). Its actual site is cytoplasmic as determined by substrate accessibility in transport deficient mutants (Weiner, 1974). A partial purification of the enzyme (Weiner and Heppel, 1972) was achieved using deoxycholate extraction followed by DEAE and phosphocellulose chromatography.

A reinvestigation of the properties of this enzyme following purification using Brij 58 was carried out by Schryvers *et al*. (1978). Electrophoresis (SDS-PAGE) revealed a single protein band of 58,000 daltons. Gel exclusion chromatography suggested that the functional enzyme existed as a dimer. Quantitation of the flavin content revealed that 0.5 moles of FAD were bound per mole of 58,000 dalton polypeptide. The activity of the enzyme with a number of electron acceptors could be influenced by the presence of detergents such as Brij 58 or Triton X-100 as well as by phospholipids. This enzyme preparation, when depleted of detergent, could reconstitute glycerol-3-phosphate dependent proline uptake in membrane vesicles of a *glp*D deficient strain.

The effects of amphipaths on the PMS coupled reduction of MTT in the detergent depleted enzyme was investigated in more detail (Robinson and Weiner, 1981). Using circular dichroism and tryptophan fluorescence, it was concluded that amphipaths probably exposed a binding site on the enzyme for PMS.

The anaerobic enzyme is characterized by its loose association with the membrane; upon cell lysis the enzyme was localized in the soluble fraction. A partial purification of the enzyme was accomplished and it was shown that the enzyme could be stimulated by FAD and FMN with FMN being the better activator (Kistler and Lin, 1982b).

The enzyme was also purified from a strain harboring a recombinant colE1 plasmid carrying the *glpA* gene (Schryvers and Weiner, 1981) and consisted of two non-identical subunits of 62,000 and 43,000 daltons. These required 20% ethylene glycol to prevent dissociation. This preparation contained one mole of non-covalent FAD and two moles of non-[illegible]on per dimer. Enzyme activity could be stimulated by exogenous FAD or FMN but the stimulation by flavins was lost if the enzyme was mildly proteolyzed or treated with sulfhydryl reagents.

SUCCINATE DEHYDROGENASE

Succinate dehydrogenase catalyzes the sixth step of the TCA cycle (the oxidation of succinate to fumarate) and can funnel reducing equivalents into the aerobic electron transport chain. The synthesis of succinate dehydrogenase is induced by aerobic growth in the absence of glucose (Ruiz-Herrera and Garcia, 1972); it is absent or found only in trace amounts in cells grown anaerobically on fumarate (van der Plas *et al.*, 1983). Succinate oxidase activity has, however, been reported in anaerobically grown cells with nitrate as terminal acceptor (Ingledew and Poole, 1984).

The succinate dehydrogenase of *E. coli*, because of its instability, has been difficult to purify and characterize. Like succinate dehydrogenases from a variety of procaryotic and eucaryotic sources, it contains a covalently bound flavin cofactor, iron and acid labile sulfur (Ohnishi and Salerno, 1982). Using immunochemical techniques, it was shown that the *E. coli* succinate dehydrogenase complex contains four polypeptides of 71,000, 26,000, 17,000 and 15,000 daltons (Condon *et al.*, 1985). The 71,000 dalton polypeptide contained the covalently bound flavin and was extremely susceptible to proteolysis. Electron paramagnetic resonance studies indicated the presence of a [2Fe-2S] cluster (centre 1), a [3Fe-xS] cluster (centre 3) and a b-type cytochrome, though the stoichiometry of this latter component was not determined. A change in the relaxation properties of centre 1 at low potentials is indicative of the presence of a third iron-sulfur centre (centre 2). The midpoint potentials for both the membrane-bound and the Lubrol-PX solubilized enzyme were +10mV, -175mV and +65mV for centres one to three respectively. Examination of the nucleotide sequences of *frd*B and *sdh*B suggest that the iron-sulfur centres might be located in these subunits (Cole, 1982; Darlison and Guest, 1984).

The Lubrol solubilized enzyme contained a b-type cytochrome tentatively identified as cytochrome b_{556}, though the identification of one of the small subunits as apocytochrome has not been achieved. The gene for cytochrome b_{556} maps to a chromosomal location close to the *sdh* operon (Murakami *et al.*, 1984). N-terminal analysis of purified cytochrome b_{556} revealed an amino acid sequence identical to that predicted by the DNA sequence for residues 4 to 27 of *sdh*C (Murakami *et al.*, 1985).

HYDROGENASE

Hydrogen metabolism occurs via two distinct pathways in *E. coli* (Sawers *et al.*, 1985). The first pathway is a hydrogen producing system present during fermentative growth. It is termed the formate hydrogen lyase system and consists of formate dehydrogenase, an unidentified electron carrier and hydrogenase (Peck and Gest, 1957). This system is responsible for the oxidation of formate to carbon dioxide and the generation of electrons coupled to the reduction of protons to evolve hydrogen gas. This latter step, catalyzed by the hydrogenase is a scalar, non-energy conserving reaction which serves to remove reducing equivalents and to help offset medium acidification (Cole, 1976). Thus, during growth on glucose in the absence of a terminal electron acceptor, *E. coli* induces the synthesis of pyruvate formate lyase which cleaves pyruvate into acetyl CoA and formate (Pecher *et al.*, 1982). The enzyme complex is repressed by nitrate or oxygen, which could serve as terminal acceptors, and is induced by formate (Wimpenny, 1969).

Hydrogen may also be used as a reductant and allow growth on malate or fumarate with succinate as the major fermentative product (Macy *et al.*, 1976). This suggested that ATP could be synthesized during cytochrome dependent electron flow from hydrogen to fumarate. Nitrate and TMAO are also able to support growth with hydrogen (Yamamoto and Ishimoto, 1978).

The respiratory hydrogenase catalyzes the oxidation of hydrogen to protons and electrons. It transfers the electrons to the quinone pool in an energy conserving, proton translocating reaction which is uncoupler sensitive and HOQNO insensitive (Jones, 1980a).

The respiratory hydrogenase was shown to be transmembranal with the use of membrane impermeant probes (Graham, 1981). It has been purified and found to be composed of two identical subunits of 56,000 daltons (Adams and Hall, 1978). The enzyme contains twelve iron and twelve acid labile sulfurs per molecule (Yoch and Carithers, 1979) and requires the presence of nickel for activity (Zinoni *et al.*, 1984).

There have long been reports of multiple forms of hydrogenase (Ackrell *et al.*, 1966). However, genetic approaches to this question suggest only that one species may exist (Glick *et al.*, 1981; Zinoni *et al.*, 1984). How these data can be reconciled with the identification of three immunologically distinct hydrogenase isoenzymes is uncertain (Ballantine and Boxer, 1985; Sawers *et al*., 1985).

FORMATE DEHYDROGENASE

The formate dehydrogenase of *E. coli* grown anaerobically on nitrate (Graham and Boxer, 1981; Enoch and Lester, 1975) has been purified and characterized. The enzyme contains iron-sulfur centres, a molybdenum cofactor and covalently linked selenium. Several oxidants can be coupled to formate dehydrogenase including oxygen, nitrate, fumarate and protons. The latter case involves the transfer of reducing equivalents from formate dehydrogenase to hydrogenase and the coupled enzymes are often termed the formate hydrogen lyase complex. Some evidence exists that there are multiple forms of formate dehydrogenase.

There are five genetic loci associated with formate dehydrogenase termed *fdh*A, B, C, D and E. Mutations at *fdh*A and B result in the loss of immunologically detectable polypeptides (Graham *et al*., 1980).

The purified enzyme consists of three subunits: the α subunit of 110,000 daltons, the β subunit of 32,000 daltons and the γ subunit with a molecular mass of 20,000 daltons. The selenium is associated with the α subunit. That the molybdenum cofactor is the same as that associated with nitrate reductase has been shown genetically using *chl* mutations (Terriere *et al*., 1981; Graham *et al*., 1980). A b-type cytochrome distinct from that associated with nitrate reductase (cytochrome b_{555}) is believed to constitute the γ subunit (Hackett and Bragg, 1982).

Topology studies have revealed that both the α and the β subunits are exposed to both cytoplasmic and periplasmic faces of the membrane (Graham and Boxer, 1981; Graham *et al.*, 1981). The location of the γ subunit remains to be elucidated.

Formate dehydrogenase is an energy conserving redox enzyme with measured proton extrusion stoichiometries of up to four and three protons per pair of electrons with oxygen and nitrate respectively (Garland *et al.*, 1975; Jones, 1980b). The use of quinones as oxidants resulted in stoichiometries approaching two protons per pair of electrons. This suggests that the formate dehydrogenase to quinone segment of the electron transport chain is proton translocating.

DIHYDROOROTATE DEHYDROGENASE

Dihydroorotate dehydrogenase catalyzes the reversible oxidation of dihydroorotate to orotate, a reaction involved in pyrimidine metabolism. In *E. coli*, the anabolic enzyme is membrane bound and repressed by pyrimidines (Taylor and Taylor, 1964). It is capable of passing reducing equivalents to both the aerobic and the anaerobic respiratory chains (Kerr and Miller, 1968; Newton *et al.*, 1971).

The purified enzyme has a native molecular weight of 67,000 and is located on the inner surface of the plasma membrane (Karibian and Couchoud, 1974; Owen and Kaback, 1978). Recently the *pyr*D gene, which codes for this enzyme, was cloned and sequenced (Jensen *et al*., 1984). The recombinant plasmid coded for a single polypeptide of 37,000 daltons indicating the native enzyme exists as a dimer of identical subunits. The presence of the plasmid in bacterial cells resulted in about 30 fold amplification in dehydrogenase activity and enabled a simpler purification to be developed (Larsen and Jensen, 1985). The purified enzyme contained about one molecule of FMN per subunit. The FMN visible absorption could be bleached by the the addition of dihydroorotate.

The anaerobic oxidation of dihydroorotate is linked to fumarate reductase in a menaquinone dependent, cytochrome independent fashion (Newton *et al*., 1971). The oxidation of dihydroorotate cannot be obligatorily linked to fumarate reduction otherwise all *frd* mutants would require uracil for anaerobic growth on glucose (Lambden and Guest, 1976).

J. ASSEMBLY OF MEMBRANE PROTEINS

The asymmetry of biological membrane components suggest that specific assembly mechanisms must exist to generate this asymmetry. The spontaneous transmembrane reorientation of membrane proteins does not occur at a measurable rate (Reithmeier, 1985).

INTRINSIC AND EXTRINSIC MEMBRANE PROTEINS

Membrane proteins have been subdivided into two classes, intrinsic and extrinsic. Extrinsic membrane proteins do not penetrate the hydrophobic core of the lipid bilayer but are associated with the surface of the membrane. This association may be mediated by intrinsic membrane proteins or by electrostatic interactions with charged phospholipids. This type of membrane protein is characterized by ready solubilization with changes in ionic strength or pH.

Intrinsic membrane proteins interact directly with the hydrophobic core of the membrane. These proteins can only be solubilized by disrupting the integrity of the membrane, usually with detergents. Small amphiphilic detergent molecules can replace the endogenous protein associated lipid while maintaining the native state of the protein. Most, if not all intrinsic membrane proteins span the membrane, allowing them to function in transport or communication processes.

STRUCTURE OF INTRINSIC MEMBRANE PROTEINS

Our knowledge of the structure of intrinsic membrane proteins has lagged far behind that of soluble proteins. This has, at least in part, due to the inaccessibility of membrane proteins to X-ray crystallography because of difficulties in obtaining suitable crystals for diffraction. Recently, however, some progress has been made. For some membrane proteins it is now possible to replace membrane lipids with small detergent molecules and then to form protein crystals (Eisenberg, 1984). This has resulted in well ordered three dimensional crystals of porin and *R. viridis* photosynthetic reaction centre (Michel, 1982). The reaction centre of *R. viridis* has provided much useful information about intrinsic membrane protein structure as well as the organization of redox groups within this complex. The centre contains four subunits; L, M, H and a c-type cytochrome (Deisenhofer *et al.*, 1984). The first three are intrinsically associated with the interior of the membrane while the cytochrome is membrane associated by virtue of its electrostatic interactions with them. At 3Å resolution, it was not possible to distinguish ordered lipid or detergent which might provide direct evidence on the position and orientation of the complex in the bilayer (Deisenhofer *et al.*, 1985). Among the three membrane embedded subunits, the authors were able to clearly define 11 long α-helices. Each of these helices is composed of 24 to 30 hydrophobic residues. The helices are roughly aligned with each other and likely span the membrane. Flat surfaces on both ends of the helices serve to interact with the membrane extrinsic portions of the H subunit and with the cytochrome. These surfaces also define the extent of the membrane.

Prior to the determination of the reaction centre structure, the prototype membrane protein was bacteriorhodopsin of *H. halobium*. This protein is a major component of the bacterial membrane, forming two dimensional arrays known as purple membrane. Using image reconstruction of low dose electron micrographs, it was possible to produce a three dimensional model for bacteriorhodopsin in the membrane with a resolution of 7Å (Unwin and Henderson, 1975). Each monomer appears to have seven transmembranal helices. Three of these are roughly perpendicular to the plane of the bilayer while the others are tilted to various degrees. The primary sequence could be arranged to cross the membrane seven times as helices of about 24 mostly hydrophobic residues (Engelman *et al*.1980).

The *E. coli* ATP synthase has also been extensively studied, but not as yet by high-resolution X-ray crystallography. Like fumarate reductase, this enzyme complex has two distinct domains, a membrane intrinsic domain, F_0, and a catalytic membrane extrinsic domain, F_1 (Futai and Kanazawa, 1983). The F_0 domain is involved in forming a proton conduction pathway through the membrane. It is composed of three non-identical subunits, all of which are thought to form at least one hydrophobic transmembranal helix. The polar residues occur outside the membrane and likely interact with the catalytic domain.

The anchor subunits of fumarate reductase are not believed to be involved in proton translocation but may serve an alternative function in coupling to the electron transport chain (see Chapter VI). Their polar residues, in analogy to the ATP synthase, may interact with the catalytic domain. The existence of distinct domains in multisubunit membrane enzyme complexes may be a common feature.

If more than one transmembrane α-helix exists, a β turn may join these segments. β turns are usually located on the surface of soluble proteins to allow for hydrogen bonding with water molecules. A similar situation is thought to occur for membrane protein β turns in that they are likely to occur outside the membrane core in a water accessible environment (Reithmeier, 1985).

Porin, which spans the outer membrane of *E. coli* form voltage dependent transmembrane channels (Schindler and Rosenbusch, 1978). This is a very polar protein which does not contain sizeable hydrophobic domains (Chen *et al.*, 1979),but consists primarily of anti-parallel β-pleated sheets oriented approximately perpendicular to the plane of the membrane (Paul and Rosenbusch, 1985). Porin seems to differ considerably from other membrane proteins in their organization, but this may be a function of the different nature of the membrane they span. Unlike the plasma membrane, the outer membrane consists of not only phospholipid but also of lipopolysaccharide.

IDENTIFICATION OF INTRINSIC MEMBRANE PROTEINS

Most membrane proteins are thought to be anchored in the membrane by hydrophobic helices, as suggested in the model for bacteriorhodopsin and as is now known in the photosynthetic reaction centre. Models for many membrane proteins have been proposed after analyzing the primary sequence with various algorithms which search for regions of high hydrophobicity. These include fumarate reductase (see Chapter IV), the human glucose transporter (which may have 12 transmembranal regions)(Mueckler *et al.*, 1985), the *E. coli* lactose carrier protein (Goldkorn *et al.*, 1983), the erythrocyte anion transport protein or Band 3 (with up to 12 helices) (Kopito and Lodish, 1985) and the calcium ATPase of the sarcoplasmic reticulum (with 10 helices)(MacLennan *et al.*, 1985).

Intrinsic membrane proteins are kept in the membrane by the interactions of hydrophobic amino acids with the lipid bilayer. Several computer algorithms have been developed to aid the investigator of membrane proteins in assigning membrane spanning segments where primary sequences are known. A recent version, one that we have used with fumarate reductase, is that devised by Kyte and Doolittle (1982). Their program systematically evaluates the hydrophobic and hydrophilic profile of a polypeptide chain. Each amino acid is assigned a value reflecting its hydrophilic and hydrophobic tendencies and the individual values of amino acids in the polypeptide chain are averaged over a chosen number of residues to give a hydropathy index. To a first approximation, the structure of a protein will be that which permits the removal of the greatest amount of hydrophobic surface and the smallest number of hydrophilic residues from accessibility to water (Kyte and Doolittle, 1982). The authors assigned each amino acid a value based on its transfer free energy between water and vapor and its distribution between the interior and exterior of proteins of known structures. Using these parameters, amino acids such as leucine, isoleucine, valine and phenylalanine are considerably hydrophobic while arginine, lysine, asparagine, aspartic acid, glutamic acid, glutamine and histidine are significantly hydrophilic.

Using the M13 coat protein, human glycophorin and the seven transmembrane α-helices of bacteriorhodopsin as models, Kyte and Doolittle suggested that any sequence of nineteen amino acids with an average hydropathy greater than +1.6 had a high probability of being a membrane spanning domain.

SIGNAL SEQUENCES AND MEMBRANE PROTEINS

E. coli is surrounded by a cytoplasmic (inner) membrane, a peptidoglycan layer and an outer membrane. A periplasmic gel is located between the inner and the outer membranes. Proteins to be localized in the outer membrane, the periplasmic gel or on the exterior surface of the inner membrane must all cross the inner membrane. All of these proteins are synthesized as precursor proteins which contain an N-terminal extension signal sequences of 15-25 residues. The amino terminal residue of the signal sequence is the initiator methionine residue which is followed by a short charged region. The centre of the signal sequence is very hydrophobic and is usually followed by a small uncharged amino acid such as glycine, alanine or serine which serves as cleavage site for the enzyme signal peptidase. The signal sequence probably adopts an α helical structure with a β turn near the cleavage site. Proteins with a signal sequence are usually synthesized on membrane associated ribosomes.

Most proteins of the inner membrane of *E. coli* are synthesized without an amino terminal signal sequence. The mechanism of their assembly into the membrane is unknown. A spontaneous partioning of hydrophobic hairpin loops into the lipid bilayer has been suggested by Engelman and Steitz (1981). These authors point out that the distribution of polar and nonpolar residues in a protein will govern the assembly of that protein and that the assembly process may be independent of receptors or transport proteins. Genetic and biochemical evidence suggests that a protein translocation complex may exist (Pugsley and Schwartz, 1985). The demonstrated importance of the amino terminal signal sequence to protein export (Wickner and Lodish, 1985), coupled with observations that some secreted proteins can be post-translationally processed and assembled (Randall and Hardy, 1984), suggest that partioning into the membrane may not require a signal sequence but that its function may be to signal a translocation complex to act upon a membrane integrated protein. In this fashion, inner membrane proteins remain in the plasma membrane because they are not recognized by a translocation complex.

K. THESIS PROBLEM

In attempting to understand the structure, organization and function of electron transport chains in general, we have focussed our efforts on a relatively simple chain as a model. The anaerobic electron transport chain from glycerol-3-phosphate dehydrogenase to fumarate reductase is composed of only three proteins, the dehydrogenase, a b-type cytochrome and fumarate reductase. The purification and partial characterization of the dehydrogenase and of the reductase had been accomplished, yet, our understanding of the ways these proteins interacted and of their topological arrangements in the inner membrane of *E. coli* remained inadequate. Our ultimate goal of reconstituting an entire coupled electron transport chain seemed unattainable in the near future.

The rapid proliferation of molecular biological technniques allowed us to expand the study of the terminal enzyme, fumarate reductase, beyond what was accessible by classical approaches. The year 1982 proved to be an eventful one since it yielded the complete sequence of the fumarate reductase operon. This was largely the work of Dr. Stewart Cole. Examination of the sequence revealed four open reading frames which could code for polypeptides of 66,000, 27,000, 15,000 and 13,000 daltons. The larger two of these polypeptides had been purified and well characterized. They formed an $\alpha\beta$ dimer capable of fumarate reduction using benzyl viologen as an artificial electron donor. This dimer behaved in all respects like a soluble protein even though it was well known that fumarate reductase required solubilizaton of the membrane for extraction. In addition, the $\alpha\beta$ dimer required the presence of high anion concentrations for maximal activity and stability. This latter observation could be rationalized if one suggested that anions might be mimicing the effects of negatively charged phospholipids. Thus, my project evolved into a study of the structure and function of the two smaller subunits and their roles in the holoenzyme.

In chapter II, we show that the 15,000 and 13,000 dalton proteins coded for in the *frd* operon are indeed subunits of the fumarate reductase holoenzyme. Our ability to show this rested on three developments. The first was the subcloning of the *frd* operon from the colicin vector, pLC16-43, into the vector pBR322. This was accomplished by Dr. Weiner and the resulting plasmid, pFRD63, proved extremely valuable in subsequent studies.

The second development, which I introduced and which has been refined by coworkers, was the utilization of acrylamide gradients in our SDS-PAGE systems. Gradient gels allowed the resolution of the *frd*C and D subunits while maintaining good resolution of higher molecular weight proteins.

From plasmid pFRD63, I was able to construct plasmid pFRD117 which coded for the A and B subunits only. This third development allowed us to identify the hydrophobic C and D subunits as membrane anchors. These subunits are responsible for the attachment of the catalytic $\alpha\beta$ dimer to the inner surface of the membrane. In their absence, the fumarate dependent benzyl viologen oxidase activity was located in the cytoplasm and not in the membrane.

Incorporation of radioactive sulfur into the cellular proteins enabled me to demonstrate that the four subunits of fumarate reductase were present in equimolar amounts in the holoenzyme. This finding was confirmed by examining staining intensities after SDS-PAGE on holoenzyme I purified from the overproducing strain.

John Robinson showed that the anchor subunits could eliminate the requirement of the dimeric enzyme for anions for maximal stability and activity

In chapter III, we further studied the roles of the anchor subunits of fumarate reductase. With the generous collaborations of Roger Bradley and Dr. Doug Scraba, I demonstrated that fumarate reductase could be visualized in electron micrographs of negatively-stained membrane preparations. The amplified membranes (strains harboring plasmid pFRD63) showed an abundance of knob and stalk structures having a diameter of approximately 4nm. In the absence of the plasmid, these structures were not observed. Using proteolysis and anti-fumarate reductase antibodies, I showed that these structures were indeed the enzyme. It was during these experiments that we noticed the presence of long tubular structures which were covered with a regular helical array of fumarate reductase molecules.

Using urea, I devised a procedure to strip the amplified membranes of most of the 69,000 and 27,000 dalton subunits while maintaining the membrane association of the anchor subunits. These stripped membranes were partially competent in reconstitution experiments where fresh catalytic dimer could be reattached to the membrane. With this reconstitution procedure, I showed that the reattached enzyme regained the properties of temperature and alkaline stability characteristic of the holoenzyme. Furthermore, upon electron microscopic examination of reconstituted membranes, we observed the reappearance of knobs previously identified as fumarate reductase on the membrane surface.

These ultrastructural studies led to our first model of the organization of the enzyme within the cytoplasmic membrane.

In chapter IV, we attempted to refine our model of the enzyme with emphasis on the organization of the anchor subunits. The DNA sequence revealed that these subunits contained approximately 70% hydrophobic residues. With algorithms developed by Kyte and Doolittle and by Dr. Wayne Anderson, we showed that each of the anchor subunits possessed three strongly hydrophobic regions of 24±4 residues in length. Further, Dr. Anderson suggested by analogy to bacteriorhodopsin, that these regions might be transmembranal α-helices. With these data, I drew two dimensional models for the arrangement of the anchor subunits in the membrane. These models predicted that the majority of polar and charged residues were connected by the three transmembranal helices in each subunit. and could be excluded from the hydrophobic core of the bilayer.

Although these models remain to be firmly established, they are likely correct. The subunits are oriented such that the hydrophilic regions face the cytoplasm. We expect these regions to be critical for the binding interactions with the catalytic dimer. Some support for the models came as a result of crosslinking studies on urea stripped membranes. I showed that both anchor subunits had lysines exposed on the same membrane face.

In chapter V, we explored the effects of overproduction of fumarate reductase on the overall membrane composition of the cell. The enzyme can account for greater than 50% of the total inner membrane protein when amplified. I measured the levels of a few membrane activities, and although the absolute levels remained approximately constant, the specific activities were markedly reduced. This reduction reflected an increase in total membrane-bound protein produced by fumarate reductase amplification. The disappearance of NADH oxidase activity from membranes when amplified levels of fumarate reductase were present was an exception to these findings. The membrane was therefore able to accommodate a large excess of fumarate reductase without displacing other membrane proteins to the cytoplasm.

Dr. Lynn Elmes determined the phospholipid and fatty acid profiles of normal and amplified membranes and showed that overproducton of fumarate reductase resulted in changes in the compositions of these components. The cell was still capable of maintaining a relatively constant lipid to protein ratio.

To further explore the tubular structures previously observed, and to determine where the extra membrane protein and phospholipid synthesized in response to plasmid pFRD63 were located, I devised a method of gently lysing cells *in situ* on carbon-coated grids prior to negative staining. This procedure, combined with thin section electron microscopy by Roger Bradley and Dr. Doug Scraba, revealed that the cell was filled with tubular structures. These appeared to be attached to the inner membrane from which, they likely originated. The appearance of these structures coincided with the time at which a soluble fumarate reductase activity could be detected. Fragmentation of the tubules to small slowly sedimenting forms probably accounted for the activity.

Chapter VI describes a novel mutant of fumarate reductase which has forced us to reexamine our views of the electron transport chain. Mutant GS19, isolated by Gillian Shaw, proved unusual in its ability to catalyze the fumarate dependent oxidation of reduced benzyl viologen while being unable to support anaerobic growth with fumarate as terminal electron acceptor. Complementation of GS19 was inconclusive and confusing, since it was observed with two different classes of plasmids. The mutant could be complemented by pFRD117 (which codes for the A and B subunits) and by pFRD704 (which codes for the C subunit only).

Using the exonuclease Bal31 to generate deletions, I constructed pFRD1144, from which Dr. Caro Condon was able to derive pFRD704. Only 36 nucleotides of the *frd*D coding sequence remained. pFRD704 proved instrumental in localizing the GS19 mutation to the C subunit of fumarate reductase. The precise sequence alteration was determined by the diligent work of Nadine Honoré and Dr. Stewart Cole. These findings alerted us to the dangers of attempting to study bacterial genetics with multicopy plasmids.

Classification of the mutant as decoupled from the electron transport chain was partially based on data comparing the benzyl viologen and DMN oxidase activities of the altered reductase. I developed an assay which used an analog of menaquinone, DMN, as the source of reducing equivalents. The mutant enzyme was unable to oxidize reduced DMN, but remained as efficient with reduced benzyl viologen. We later postulated that DMN and the physiological electron donor used the same site on the enzyme. Quinacrine quenching results and coupled assay systems developed by Dr. Joel Weiner further supported the hypothesis that the mutant enzyme was unable to communicate with the electron transport chain. We are investigating the possibility that this may be related to the enzyme's inability to accept electrons in pairs as might be expected with quinone donors.

Our ability to monitor fumarate dependent quinone oxidation revealed to us the vital role that the C and D subunits play in electron transport: they are not merely passive membrane anchors uninvolved in the redox reactions of the complex.

EPR studies by Dr. Richard Cammack revealed abnormal redox centres in the mutant enzyme. The covalent FAD was more readily stabilized as a flavin radical in the mutant enzyme and reduction of iron-sulfur cluster 1 by dithionite was unusually slow. It seemed remarkable that such effects could be the result of a mutation in *frd*C.

The properties of GS19 have yet to be fully explored, but the mutant has already forced us to reassess some of the dogma surrounding fumarate reductase. The anchor subunits have been elevated to active participation in the catalytic process. More importantly, the enzyme's ability to oxidize a menaquinone analog forces us to question the role and location of the b-type cytochrome included in this respiratory chain.

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II. IDENTIFICATION OF MEMBRANE ANCHOR POLYPEPTIDES OF *Escherichia coli* FUMARATE REDUCTASE[1]

A. INTRODUCTION

Fumarate reductase serves as the terminal electron transfer enzyme when *Escherichia coli* is grown anaerobically on glycerol plus fumarate. We have purified this membrane-bound enzyme and extensively characterized its enzymatic, chemical and physical properties (Dickie and Weiner, 1979; Robinson and Weiner, 1981; Weiner and Dickie, 1979). The enzyme we have described is an $\alpha\beta$ dimer of 69,000 and 27,000 dalton subunits. Recently, the nucleotide and thus inferred amino acid sequence of the *frd*A gene (69,000 dalton catalytic subunit)(Cole, 1982) and the *frd*B gene (27,000 dalton non-heme iron containing subunit)(Cole *et al.*, 1982) have become available. Examination of the nucleotide sequence of the HindIII fragment coding for the *frd*A and *frd*B genes indicated the presence of two additional sequences coding for two small polypeptides of 15,000 and 13,000 daltons in the *frd* operon originally termed G15 and G13 and now referred to as *frd*C and *frd*D respectively. A portion of *frd*D appears to overlap the *amp*C gene (Grundström and Jaurin, 1982). The amino acid compositions of the *frd*C and *frd*D polypeptides are extremely hydrophobic and relatively basic. It seemed possible that these two polypeptides were part of a fumarate reductase holoenzyme. In this paper, we show that *frd*C and *frd*D are expressed in concert with *frd*A and *frd*B, are components of the holoenzyme and serve as the membrane anchors for the catalytic and iron containing subunits.

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[1] A version of this chapter has been published. Lemire, B.D., J.J. Robinson and J.H. Weiner. 1982. *J. Bacteriol.* *152*:1126-1131.

B. MATERIALS AND METHODS

Strains and Plasmids

Escherichia coli HB101 is F^-, *hsd*R, *hsd*M, *pro*, *leu*, *gal*, *lac*, *thi*, *rec*A and *rps*L and was provided by Dr. G. McFadden of this department. The plasmid pLC16-43 has been described previously (Lohmeier *et al.*, 1981). pBR322 was isolated from HB101/pBR322 after chloramphenicol amplification (Cole and Guest, 1980; Guerry *et al.*, 1973).

Construction of pFRD63 and pFRD117

To construct pFRD63 220 ng of pBR322 and 610 ng of pLC16-43 were digested with 5 units of the restriction endonuclease HindIII for 2 hr at 37°C as described previously (Schryvers and Weiner, 1982). The digested material was ligated with 1 unit of T4 DNA ligase (Schleif and Wensink, 1981) and the DNA isolated by ethanol precipitation at -70°C. For transformation ligated DNA (44 ng of pBR322) was incubated with 0.3 ml of competent HB101 cells (17) and plated on Luria broth agar plates (Scleif and Wensink, 1981) containing 100 ug/ml ampicillin. Colonies were screened for the appropriate plasmid by the miniscreening procedure of Birnboim and Doly (Birnboim and Doily, 1979) and the structure was confirmed by restriction endonuclease mapping (Figure II.1).

To construct pFRD117 we digested 690 ng of pFRD63 with restriction endonuclease SalI and ligated with 1 unit T4 DNA ligase, purified the ligated DNA and transformed *E. coli* HB101 as described above. The appropriate plasmid was detected by miniscreening and confirmed by restriction endonuclease mapping (Figure II.1).

Preparation of Cell Envelope Fraction, Cytoplasmic Fraction and Triton X-100 Extracts

Cells were grown aerobically or anaerobically on glycerol-fumarate (GF) medium (Spencer and Guest, 1974) to late stationary phase, washed once with 50 mM Tris-HCl, pH 8.0 resuspended to 0.2 gm per ml, and lysed by two passages through a French Pressure Cell (Aminco Inst. Co., Silver Spring, Md.) at 16,000 psi. The cell envelopes were obtained by centrifuging the crude lysate at 11,000 xg for 10 min at 4°C and the supernatant spun at 160,000 xg for 60 min at 4°C. The supernatant of the high speed spin is referred to as the cytoplasmic fraction. The membranes were homogenized in 50 mM Tris-HCl, pH 8.0 containing 0.15 M KCl, 0.25 M sucrose, 10 mM EDTA and spun at 160,000 xg for 60 min at 4°. The membranes were homogenized in 50 mM Tris-HCl and stored at a concentration of 7 mg protein/ml at -70°C. To

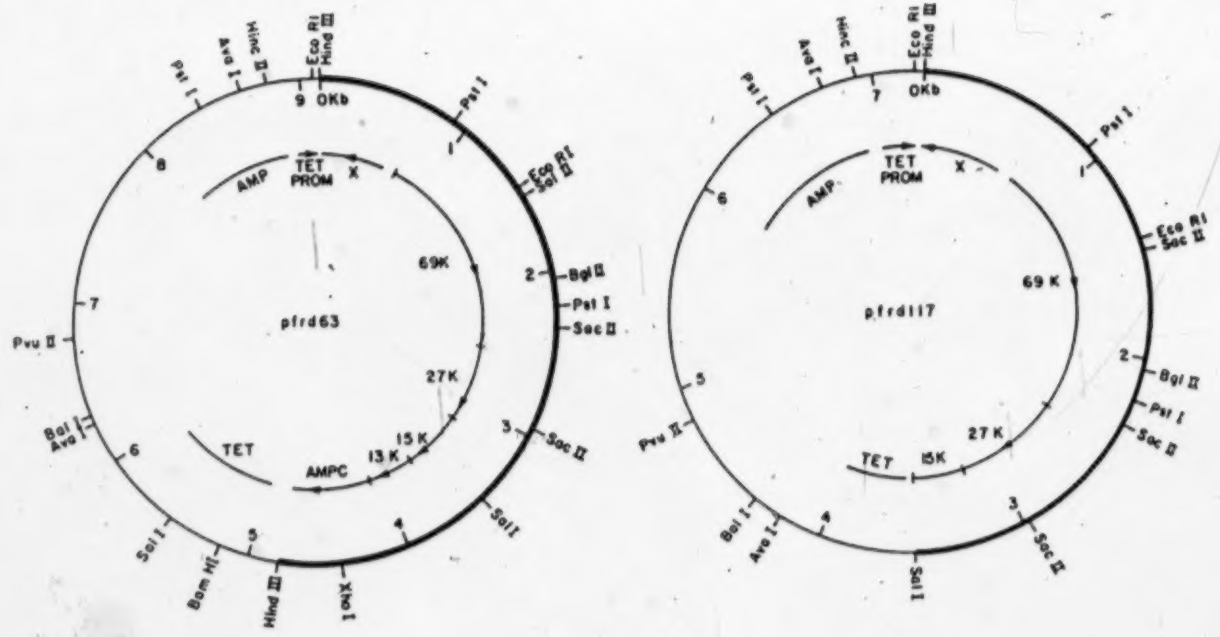
Eco RI
Hind III
Hinc II
Ava I
Pst I
9
0Kb
Pst I
1
Eco RI
Sal I
8
AMP
TET
PROM
X
69K
2
Bgl II
Pst I
Sac II
7
Pvu II
pfrd63
27K
15K
13K
TET
AMPC
3
Sac II
Bal I
Ava I
6
Sal I
5
Bam HI
Hind III
Xho I
4
Sal I
Eco RI
Hind III
Hinc II
Ava I
Pst I
7
0Kb
Pst I
1
Eco RI
Sac II
6
AMP
TET
PROM
X
69 K
pfrd117
2
Bgl II
Pst I
Sac II
5
Pvu II
27 K
TET
15K
3
Sac II
4
Bal I
Ava I
Sal I

Figure II.1 Restriction endonuclese maps of pFRD63 and pFRD117. These maps were determined by multiple restriction enzyme digests as described previously (Lohmeier *et al*., 1981; Schryvers and Weiner, 1982). pFRD63 is 9.1 Kb and pFRD117 is 7.2 Kb. AMP refers to the ampicillin resistance gene of pBR322, AMPC refers to the *E. coli* chromosomal ampicillin resistant gene adjacent to the *frd* operon.

prepare the Triton X-100 extract, Triton was added to a final concentration of 1% (w/v) and incubated for 1 hr at 4°C. The Triton solubilized protein (4 mg/ml) was recovered after removal of insoluble debris by centrifugation at 48,000 xg for 60 min at 4°C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Polyacrylamide gradient slab gels were run in the buffer systems described by Laemmli (Laemmli, 1970) or Weber and Osborn (Weber and Osborn, 1969) as indicated in the Results. Molecular weights were determined using both the high and low molecular weight marker mixes prepared by BRL (Bethesda, Md.). Electrophoresis was at 30 mA until the tracker dye, bromophenol blue, reached the bottom of the slab. Gels were stained in 0.1% Coomassie Blue in 25% isopropanol, 10% acetic acid, 0.1% cupric acetate and destained in 25% methanol, 10% acetic acid and scanned on a Gilford Model 2600 spectrophotometer equipped with a scanning attachment and integrator.

Labelling Cells with ^{35}S Sulfate

HB101/pFRD63 was grown anaerobically on sulfate-free glycerol-fumarate medium in the absence of added casamino acids to which was added 10 μCi/ml ^{35}S sulfate. The final concentration of sulfate was adjusted to 120 μM with unlabe~~ll~~ed sodium sulfate. Cells were harvested in late stationary phase and washed in 200 mM sodium phosphate pH 6.8. The cells were lysed and membranes prepared as described above. 150,000 counts were applied to a 10-25% Laemmli gel. Destained gels were sliced into 1 mm slices; each slice was incubated in a scintillation vial for 4 hr at 50°C with 0.5 ml NCS solubilizer (Amersham Corporation, Arlington Heights, Il.) containing 6% water. The vials were cooled to room temperature and 17 μl of glacial acetic acid followed by 10 ml of Aquasol (New England Nuclear, Boston, MA.) were added. Then the vials were shaken and counted.

Purification of Fumarate Reductase

The two subunit form of fumarate reductase was purified as described previously (Dickie and Weiner, 1979). The holoenzyme form was purified by applying 100 ul (4 mg/ml) of the Triton X-100 extract (specific activity, 170 units/mg) to a 3.6 ml, 5-20% linear sucrose gradient prepared in 0.1 M potassium phosphate, pH 6.8 and 0.2% Triton X-100. The gradients were spun for 16 hr at 50,000 rpm, 4°C in a Beckman SW56 rotor. Gradients were eluted from the bottom and assayed for activity. Recovery was routinely 80% and the purity of the peak fractions (specific activity, 220 units/mg) was judged to be greater than 95% by polyacrylamide gel electrophoresis.

Assay of Fumarate Reductase

Fumarate reductase was assayed by following the oxidation of reduced benzyl viologen as previously described

(Dickie and Weiner, 1979) in either 0.2 M potassium phosphate, pH 6.8 (anion buffer) or 50 mM HEPES, pH 6.8 (anion-free buffer) as detailed in the Results. One unit of activity corresponds to one µmole of benzyl viologen oxidized per minute at 24°C.

Stability of Fumarate Reductase to Alkaline pH

The appropriate enzyme fraction was applied to a 2 ml column of Sephadex G-25 equilibrated with 20 mM Tris-HCl pH 8.6. The column was eluted with equilibration buffer and active fractions combined. This operation took 8-9 min. At 10 min an aliquot was assayed for fumarate reductase activity and this value taken as 100%.

Sources of materials

^{35}S sodium sulfate (906 mCi/mmole) was from New England Nuclear (Boston, MA). Restriction endonucleases were from BRL (Bethesda, Md.). T4 DNA ligase was from Boehringer Mannheim, (Montreal, P.Q.). All other chemicals were from Sigma Chemical Co. (St. Louis, Mo.).

C. RESULTS

Expression of Fumarate Reductase in Stains Carrying pFRD63 and pFRD117

In a previous paper (Lohmeier *et al.*, 1981), we showed that the expression of the 69,000 and 27,000 dalton polypeptides of fumarate reductase correlated with enzyme activity and that these two bands were the major polypeptide components of the cytoplasmic membrane. The 15,000 and 13,000 polypeptides were not visualized in those earlier experiments because they migrated with the tracker dye.

To examine the expression and role of *frd*C and *frd*D we prepared French Press membrane envelopes from cells carrying the plasmid pBR322 or the recombinant plasmid pFRD63 or pFRD117. pFRD63 carries a HindIII fragment coding for *frd*A, *frd*B, *frd*C and *frd*D (Cole, 1982; Cole *et al*., 1982). In pFRD117, we have deleted the segment between the SalI site in pBR322 and that within the 15K gene. Thus, pFRD117 carries only *frd*A, *frd*B and a fragment of *frd*C. Table II.1 summarizes the activity data we observed. It can be seen that the presence of pFRD63 results in a 20-fold amplification of activity when the cells are grown anaerobically. The appearance of activity in the soluble fraction is only observed after the membrane becomes saturated with fumarate reductase. This occurs when a specific activity of 60 to 70 units/mg is reached. During aerobic growth with a recombinant plasmid we still observe significant activity but it is fifteen-fold lower than under anaerobic conditions. The aerobic expression may be due to the titration of a repressor protein (Clewell, 1972).

A similar experiment was carried out using pFRD117. The recombinant plasmid which only carries the *frd*A and *frd*B genes. This plasmid also results in a substantial

Table II.1

Aerobic and anaerobic expression of fumarate reductase

	Aerobic growth				Anaerobic growth			
	Membranes		Cytoplasm		Membranes		Cytoplasm	
Strain	Sp.Ac.	Units	Sp.Ac.	Units	Sp.Ac.	Units	Sp.Ac	Units
HB101(pBR322)	0.2	0.9	<0.1	<1.0	5.3	14.3	<0.1	<1.0
HB101(pFRD63)	3.7	15.2	<0.1	<1.0	66.8	294.0	9.9	128.0
HB101(pFRD117)	0.4	1.4	2.6	39.0	7.0	19.5	78.8	945.0

Cells were grown either aerobically or anaerobically to stationary phase in glycerol fumarate medium. Activity was assayed in 0.2 M potassium phosphate, pH 6.8 and is expressed as micromoles of reduced benzyl viologen oxidized per minute. Specific activity (Sp.Ac.) is expressed as units per milligram of protein.

amplification of fumarate reductase activity but interestingly the majority of the activity is in the soluble fraction. Thus it appears that in the absence of the *frd*C and *frd*D polypeptides, the two large subunits do not associate with the membrane. Considering this in light of the very hydrophobic composition of both the 15,000 and 13,000 dalton polypeptides, we have proposed that these polypeptides serve as the membrane anchors for the catalytic part of the enzyme.

Expression and Localization of *frd*C and *frd*D

The membrane envelopes from HB101/pFRD63 were examined on SDS-PAGE using the Laemmli buffer system and 10-25% linear polyacrylamide gradients (Laemmli, 1970) (Figure II.2). Four predominant bands at 69,000, 25,000, 15,000 and 14,000 can be seen. These molecular weights do not correspond exactly to the molecular weights estimated from the sequence. The 27,000 dalton polypeptide migrates at a position corresponding to 25,000 and the 13,000 polypeptide at 14,000. When the area under each stained peak was integrated, a mole ratio of 1:1.1:1.1:1.2 was obtained for these four bands. The corresponding mole ratio as determined with ^{35}S sulfate labelled proteins is 1:1.3:1.2:1.0 (Table II.2) When a Triton X-100 extract was prepared from these envelopes to remove outer membrane polypeptides (Schnaitman, 1970) and subjected to SDS-PAGE, these four polypeptides were the predominant components of the

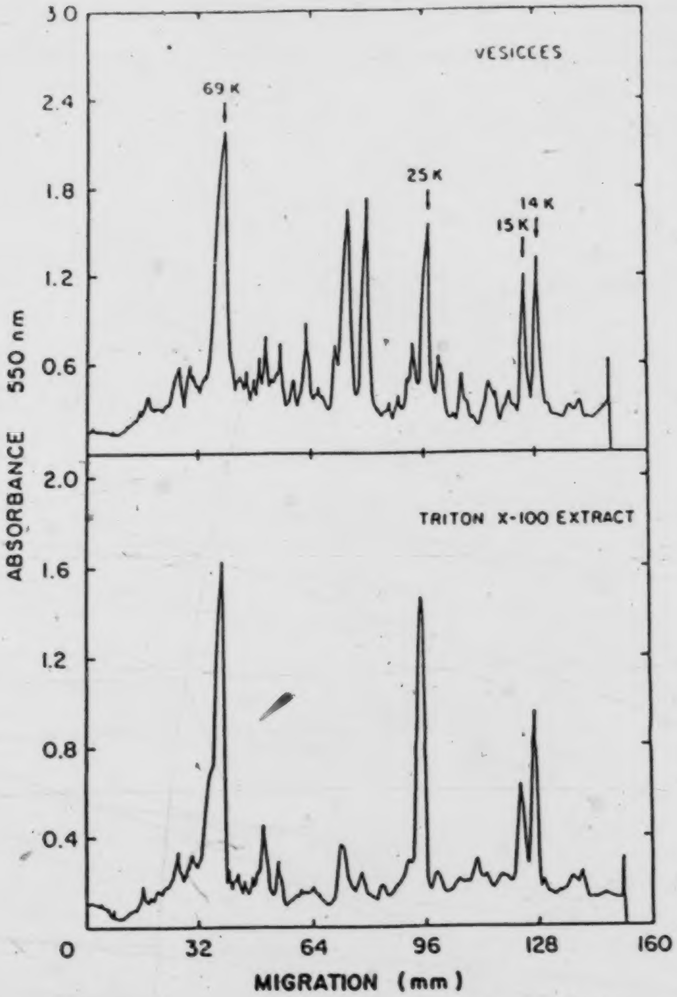

Figure II.2 Densitometric scans of SDS-PAGE patterns of vesicles and Triton X-100 extracts. Densitometric scans of the SDS-PAGE pattern of (a) French press membrane vesicles and (b) Triton X-100 extract of HB101/pFRD63. 200 μg of membrane protein was applied to a 10-25% linear gradient polyacrylamide resolving gel in the Laemmli buffer system. Molecular weights were determined using the following markers: alkaline phosphatase, 92,000; bovine serum albumin, 68,000; ovalbumin, 43,000; chymotrypsinogen, 25,000; β-lactoglobin, 18,400; cytochrome c, 12,300; bovine trypsin inhibitor, 6,200.

extract. In fact, they comprised about 75% of the total protein in the fraction and were present in 1:1:0.8:1.1 mole ratios based on staining intensity (Figure II.2). A similar experiment was done with cells grown aerobically, all four bands were diminished about ten-fold in intensity (data not shown). If cells carrying pBR322 were used, these four polypeptides could barely be detected.

In a similar experiment, membranes and cytoplasm were prepared from HB101/pFRD117 which does not code for *frd*C and *frd*D. As can be seen in Figure II.3 very little of the polypeptides corresponding to fumarate reductase could be found associated with the membrane. However, when the cytoplasmic fraction was examined, the 69,000 and 27,000 dalton bands were clearly observed (Figure II.3), in agreement with the activity measurements. This experiment confirms that expression of *frd*C and *frd*D is required for the 69,000 and 27,000 dalton polypeptides to bind to the membrane.

In agreement with the observation of Grundström and Jaurin (Grundström and Jaurin, 1982), we observed anomolous behaviour of *frd*C and *frd*D on different SDS-polyacrylamide gel systems. When the Weber and Osborn buffer system (Weber and Osborn, 1969) was used, *frd*C and *frd*D could not be resolved.

Properties of Fumarate Reductase Holoenzyme

In a recent paper (Robinson and Weiner, 1981), we reported that the two subunit catalytically active form of fumarate reductase was dependent on anions (e.g. phosphate) for optimal activity. Additionally, anions stabilized the

Table II.2

Determination of the fumarate reductase subunit mole ratio

Subunit	Number of cys + met	cpm in subunit peak	cpm per cys + met	Mole ratio
69,000	30	6,044	201	1.0
27,000	20	5,331	267	1.3
15,000	4	950	238	1.2
13,000	7	1,403	200	1.0

The number of sulfur containing residues (cysteine + methionine) in each subunit was determined from the DNA sequence (Cole, 1982; Cole *et al.*, 1982; Grundström and Jaurin, 1982). cys=cysteine; met=methionine.

enzyme against alkaline pH denaturation and thermal inactivation. Based on a comparison of these parameters for the membrane-bound versus purified forms we proposed that anions converted the solubilized enzyme to a state which more closely resembled the membrane-bound state.

Interestingly, the four subunit holoenzyme form of the enzyme was not dependent on anions for activity (Table II.3) and was stable at alkaline pH (Figure II.4). It was however as thermolabile as the two subunit form and this thermolability could be prevented by anions (Figure II.5). For these studies we used either the Triton X-100 extract, estimated to be 75% pure by integration of Coomassie Blue stained polyacrylamide gels, or enzyme purified to 95% homogeneity by centrifugation through 5 to 20% sucrose gradients . It should be noted that the specific activity of the holoenzyme was lower than expected, based on a comparison of staining intensity with our studies of the two subunit form.

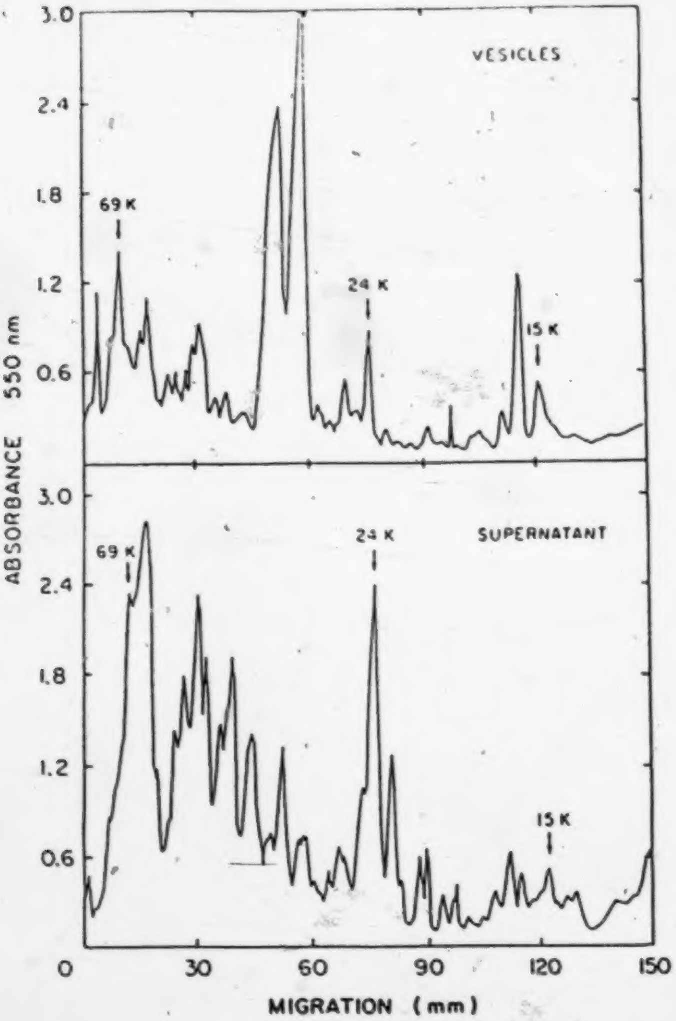

Figure II.3 Densitometric scans of SDS-PAGE patterns of membrane and cytoplasmic fractions. Densitometric scans of the SDS-PAGE pattern of (a) French press membrane vesicles and (b) cytoplasmic fraction of HB101/pFRD117. 200 μg of protein was applied to each well and conditions were as described in Figure II.2.

The specific activity of the Triton X-100 extract was only 170 units/mg whereas we would have expected a specific activity of about 400 units/mg based on the apparent purity. It may be that a fraction of the enzyme is catalytically inactive and this is currently under study.

Table II.3

Stimulation of fumarate reductase activity of potassium phosphate

Enzyme	Assay A Sp.Ac.	Assay B Sp.Ac.	B/A ratio
Two subunit form	100	550	5.50
Four subunit holoenzyme	172	164	0.95
Membrane bound form	62	68	1.09

Enzyme was assayed in either A) 25 mM HEPES, pH 6.8 or B) 0.2 M potassium phosphate, pH 6.8. Sp.Ac.=specific activity in units per milligram of protein.

Loss of the 15,000 and 13,000 Polypeptides During Purification of Catalytically Active Fumarate Reductase

It was clear that the purified catalytically active fumarate reductase we have been characterizing is composed of only two subunits of 69,000 and 27,000 daltons and does not contain any material in the molecular weight range corresponding to *frd*C or *frd*D (16). As our purification involves only two steps; ammonium acetate precipitation and hydrophobic exchange chromatography on Phenyl Sepharose we decided to see which of these steps removed the two small polypeptides. They were not dissociated by the ammonium acetate treatment. However, on a Phenyl Sepharose column the 15,000 and 13,000 polypeptides bound extremely tightly when applied in 50 mM Tris-HCl, pH 6.8 containing 17% ammonium acetate and did not elute with the enzyme activity when the column was developed with a linear gradient of 17% to 2% ammonium acetate in 0.5% sodium cholate and 50 mM Tris-HCl. They could be quantitatively eluted with a 1%

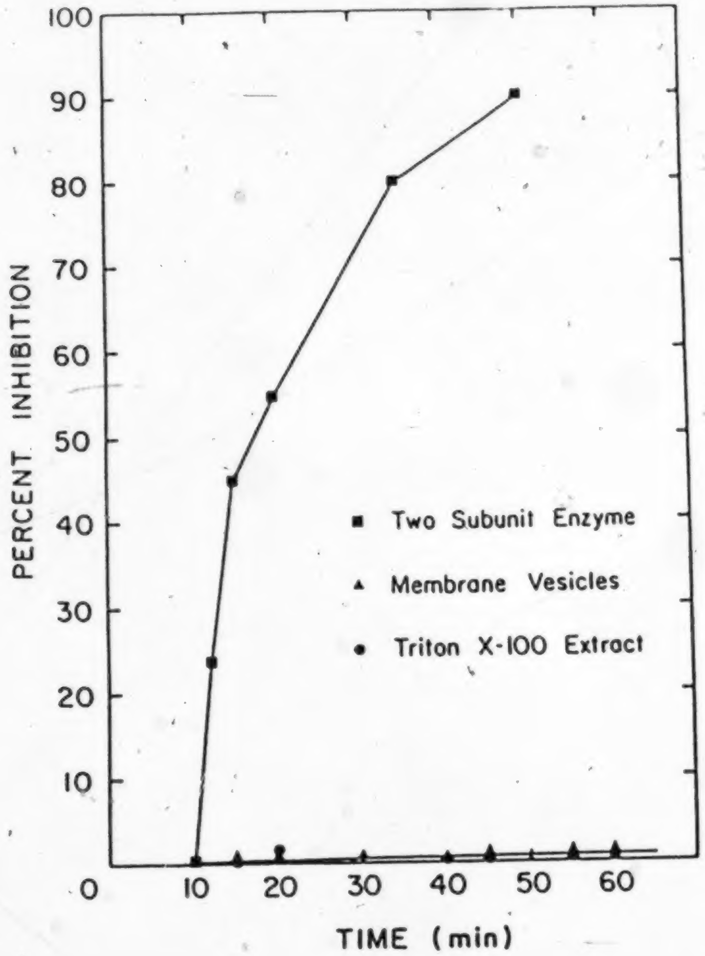

Figure II.4 Stability of fumarate reductase to alkaline pH. Incubations were at 24°C in 20 mM Tris-HCl, pH 8.6. The membrane-bound enzyme (▲) was 6.6 mg/ml and had a specific activity of 59 units/mg. The four-subunit holoenzyme (Triton X-100 extract) (●-●) was 1.65 mg/ml, specific activity of 167 units/mg protein. The 2 subunit enzyme (■-■) was 2.8 mg/ml, specific activity 550 units/mg protein.

Triton X-100 wash of the column. However, this fraction was heavily contaminated with other proteins retained by the Phenyl Sepharose column which co-eluted.

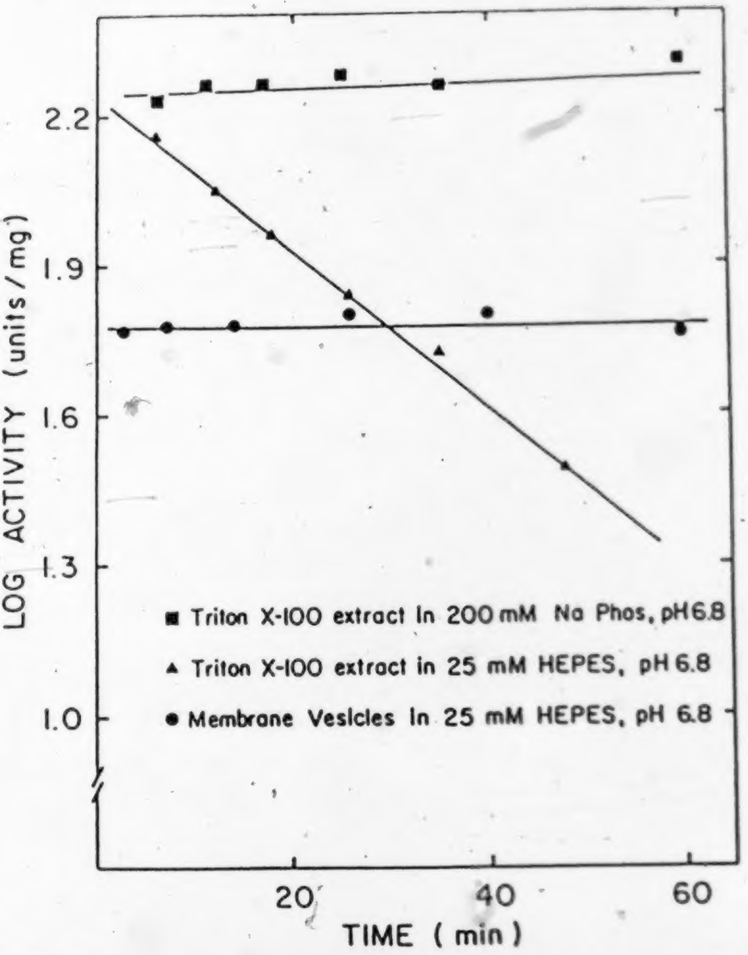

Figure II.5 Thermostability of membrane bound and Triton X-100 (holoenzyme) extracted fumarate reductase. The membrane vesicles were 6.7 mg/ml, specific activity 58 units/mg. The Triton X-100 extract in 25 mM Hepes, pH 6.8, was 1.4 mg/ml, specific activity 180 units/mg protein, while in 200 mM sodium phosphate, pH 6.8 it was 0.7 mg/ml, specific activity 171 units/mg protein. All incubations were at 45°C.

D. DISCUSSION

In this paper, we have shown that the two small polypeptides of 15,000 and 13,000 daltons coded within the *frd* operon are part of a 4-subunit holoenzyme form of fumarate reductase. This conclusion is based on the co-expression, co-extraction and co-purification of all four subunits, as well as activity studies. The sequences of *frd*C and *frd*D show long stretches of hydrophobic amino acids (Grundström and Jaurin, 1982) and we have proposed that they function, at least in part, to anchor the catalytic (69,000 dalton) subunit and the non-heme iron containing (27,000 dalton) subunit to the cytoplasmic membrane. This conclusion was drawn from studies with the plasmid pFRD117. This plasmid carries only the *frd*A and *frd*B genes and fumarate reductase is found primarily in the cytoplasmic fraction in a strain harboring this plasmid.

These two small polypeptides also serve a role in maintaining the structure of the two large subunits. This was clearly shown by the anion independence and alkaline stability of the holoenzyme form of fumarate reductase compared to the two-subunit form. In these respects the holoenzyme form closely resembles the membrane-bound activity. The picture is not complete however, as both the 2-subunit and holoenzyme forms are thermolabile, in contrast to the membrane-bound form. It is interesting that anions stimulate and stabilize the 2-subunit fumarate reductase because both the 15,000 dalton and 13,000 dalton polypeptides are rather basic proteins (Grundström and Jaurin, 1982). The explanation for this dichotomy is not clear.

Fumarate reductase is not unique in having membrane anchor polypeptides. Beef heart succinate dehydrogenase appears to have two hydrophobic polypeptides which may serve a similar role (Girdlestone *et al.*, 1981; Merli *et al.*, 1979). The hydrophobic F_0 portion of F_0/F_1 ATPase appears to bind the F_1 portion to the membrane in addition to its role in proton translocation (Fillingame, 1980).

A more detailed study of the interactions between the anchor polypeptides and the catalytic subunits is presently being undertaken.

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III. STRUCTURE OF FUMARATE REDUCTASE ON THE CYTOPLASMIC MEMBRANE OF *ESCHERICHIA COLI*

A. INTRODUCTION

Fumarate reductase serves as the terminal electron transfer enzyme in a very simple electron transport chain induced when *E. coli* is grown anaerobically on glycerol and fumarate(Haddock and Jones, 1977). This enzyme is a membrane-bound tetramer consisting of four non-identical subunits; a 69 Kd subunit, *frd*A which contains a covalently-bound flavin adenine dinucleotide cofactor (Weiner and Dickie, 1979), a 27 Kd subunit, *frd*B containing a non-heme iron sulfur centre (Cole *et al.*, 1982) and two small very hydrophobic subunits (*frd*C and *frd*D) of 15 Kd and 13 Kd (Lemire *et al.*, 1982). The latter two subunits have been shown to anchor the catalytic 69 Kd and 27 Kd subunits to the cytoplasmic membrane (Lemire *et al.*, 1982).

Fumarate reductase can be purified either as a catalytically active dimer composed of the larger two subunits or as a tetrameric holoenzyme (Dickie and Weiner, 1979; Lohmeier *et al.*, 1981). The properties of the holoenzyme and the two subunit enzyme differ in a number of respects. The dimer requires anions for optimal activity whereas the holoenzyme is anion independent (Robinson and Weiner, 1982). Additionally, the dimer is considerably more heat and

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[1] A version of this chapter has been published. Lemire, B.D., J.J. Robinson, R.D. Bradley, D.G. Scraba and J.H. Weiner. 1983. *J. Bacteriol.* *155*:391-397.

alkaline labile than the holoenzyme (Lemire *et al.*, 1982).

As reported in this paper, we have extended our understanding of the structure of fumarate reductase and shown that it can be visualized on the inner surface of cytoplasmic membranes containing greatly amplified levels of the enzyme, which were isolated from a strain which carries a multicopy recombinant plasmid coding for the fumarate reductase operon. The holoenzyme is composed of a membrane extrinsic catalytic domain and a membrane intrinsic anchor domain. These domains can be dissociated and functionally reassociated in everted membrane vesicles.

B. MATERIALS AND METHODS

Strains and Plasmids

E. coli HB101 is F^-, *hsd*R, *hsd*M, *pro*, *leu*, *gal*, *lac*, *thi*, *rec*A and *rps*L. The plasmids pBR322, pFRD63 and pFRD117 have previously been described (Lemire *et al.*, 1982).

Preparation of Everted Inner Membranes and Cytoplasmic Fractions

Cells were grown anaerobically on glycerol-fumarate medium to late stationary phase (Spencer and Guest, 1974). Purified everted inner membranes prepared by French pressure cell lysis (Aminco Inst., Rockville, Md.) were isolated by the procedure of Yamato *et al.* (Yamato *et al.*, 1978), except that the dialysis step and final wash were omitted.

Preparation of the cytoplasmic fraction from HB101/pFRD117 has been described previously (Lemire *et al.*, 1982).

Sodium Dodecyl Sulfate Polyacrylamide Gel

Electrophoresis on 10-25% acrylamide gradient gels was carried out using the Laemmli buffer system (Laemmli, 1970).

Purification of Fumarate Reductase and Assay of Activity

The two subunit form of fumarate reductase was purified by Triton X-100 extraction and hydrophobic exchange chromatography as previously described (Dickie and Weiner, 1979). The activity was assayed by following the oxidation of reduced benzyl viologen (Dickie and Weiner, 1979). One unit of activity corresponds to 1 µmole of benzyl viologen oxidized per minute at 24°C. The holoenzyme form was purified by Triton X-100 extraction and sucrose gradient centrifugation as previously described (Lemire *et al.*, 1982).

Chymotrypsin Treatment of Everted Inner Membranes

Inner membranes were suspended in 0.2 M NaPi pH 6.8 to a final protein concentration of 5.3 mg/ml. The membranes were digested for 2 min at 30°C with N-α-p-tosyl-L-lysine chloromethyl ketone-treated chymotrypsin in 0.2 M NaPi, pH 6.8, at a protein/chymotrypsin ratio of 10/1. The digestion was stopped by the addition of 10 µg of N-tosyl-L-phenylalanyl chloromethyl ketone in 3 µl of isopropanol to 200 µl of digestion mixture which contained 0.9 mg of membrane protein. The membranes were harvested by centrifugation at 160,000 xg for 10 min in a Beckman Airfuge and washed twice in 50 mM NaPi pH 6.8.

Urea Wash of Everted Inner Membranes

Everted membranes were stripped of the 69 and 27 Kd subunits as follows. Typically 4.2 mg (42 mg/ml) of inner membrane protein from HB101/pFRD63 or HB101/pBR322 were diluted with 9 volumes of 50 mM Tris·HCl, pH 8.0. 9 M urea was added to a final concentration of 6 M and the mixture was incubated at 0°C for 4 hours with occasional mixing. The membranes were recovered by centrifuging at 160,000 xg for 60 minutes at 2°C. The membrane pellet was resuspended in 50 mM NaPi, pH 6.8 and sonicated at 4°C for 90 seconds at a power setting of 40 watts in a Branson Probe Type Sonicator. The membranes were stored at -70°C.

Reconstitution of Stripped Inner Membranes

For titration experiments, 10 μg of stripped inner membrane protein were incubated with 2 volumes of 1 M NaCl, 2 volumes of 10 mM DTT and up to 20 volumes of HB101/pFRD117 cytoplasmic fraction (5.6 mg/ml) or purified two-subunit fumarate reductase (8.9 mg/ml). For alkaline and temperature stability experiments 40 to 80 μg of membrane protein were incubated with 15 volumes of HB101/pFRD117 cytoplasm. All reconstitution experiments were incubated at 0°C for 60 minutes, and centrifuged at 140,000 xg for 15 min at

24°C in a Beckman airfuge. The membrane pellets were resuspended in 200 mM NaPi, pH 6.8 for titration experiments or in 10 mM NaPi, pH 6.8 for other experiments.

Temperature and pH Stability Experiments

HB101/pFRD117 cytoplasmic fraction was desalted on a 2 ml column of Sephadex G-25 equilibrated in 10 mM NaPi, pH 6.8. The column was eluted with equilibration buffer and colored fractions combined. At the initiation of the experiment an equal volume of 0.1 M Tris-HCl, pH 8.8, was added to the desalted cytoplasmic fraction and the reconstituted inner membranes (1.4 mg protein) in 10 mM NaPi pH 6.8. The samples were incubated at 0°C and aliquots assayed at indicated times.

For temperature stability experiments, reconstituted inner membrane and HB101/pFRD117 cytoplasmic fractions were prepared as for pH stability studies. Samples were immersed in a circulating water bath at 45°C.

Electron Microscopy

Membranes at a protein concentration of 0.7 mg/ml in 50 mM NaPi, pH 6.8, were absorbed to hydrophilic carbon films and negatively stained with 1% sodium phosphotungstate (pH 7.0). Photographs were obtained in a Phillips model EM300 electron microscope operated at 80 kV. Digestions with proteinase K (E. Merck, Biochemicals) were performed for 18 hrs at 25°C with a protease concentration of

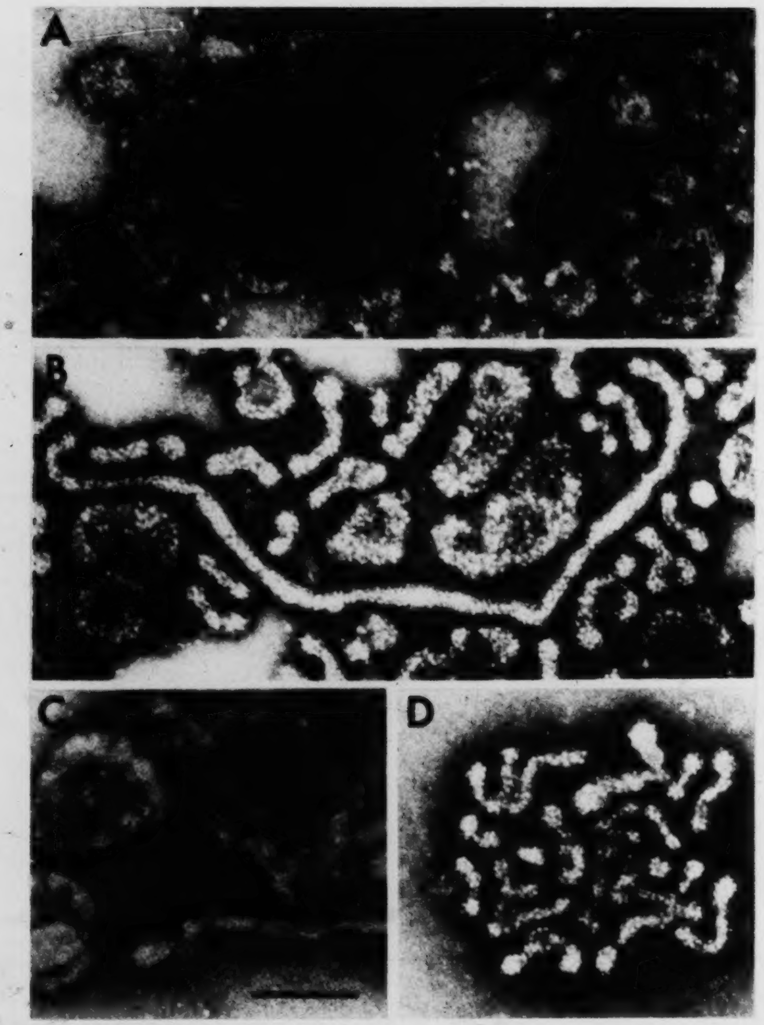

Figure III.1 Electron Micrographs of normal and amplified membranes. Electron micrographs of: (A) normal *E. coli* inner membranes (HB101/pBR322); (B) inner membranes from *E. coli* carrying plasmid pFRD63; (C) same as in (B), but following overnight incubation at room temperature with proteinase K; (D) same as in (B), but following the addition of antibody (IgG fraction) against the catalytic dimer of fumarate reductase. The bar represents 100nm.

50 ug/ml. Anti-fumarate reductase antibody was prepared in rabbits and the IgG fraction isolated by the procedure of Broome and Gilbert (Broome and Gilbert, 1978). Membranes were incubated with 0.1 mg/ml IgG protein at 25°C for 18 hrs

prior to electron microscopy.

C. RESULTS

Visualization of Fumarate Reductase

We have previously shown that when fumarate reductase is induced by anaerobic growth in the presence of fumarate, the cytoplasmic membrane from strain HB101/pFRD63 contains dramatically amplified levels of the enzyme (Lemire *et al.*, 1982). Negatively stained preparations of these everted membrane vesicles, prepared by French Press lysis, were examined in the electron microscope. The cytoplasmic surface appeared to be covered with a high density of knob-like structures. In many of the micrographs the knobs appeared attached to the membrane by short stalks (Figure III.1b) These knobs were about 4.3 nm ± 0.3 nm in diameter and the stalks about 1 nm in length. Evidence that the knob and stalk structure may, in fact, be fumarate reductase came from the following studies. Membranes isolated from strain HB101/pBR322 containing the unamplified (normal) level of fumarate reductase had many fewer knobs on the surface. (Figure III.1a) Treatment of the membranes with Proteinase K destroyed all surface structure (Figure III.1c) and SDS polyacrylamide gel electrophoresis of the resulting vesicles indicated that the 69 Kd and 27 Kd subunits were absent (data not shown). Antibody prepared against the 69 and 27 Kd subunits of fumarate reductase

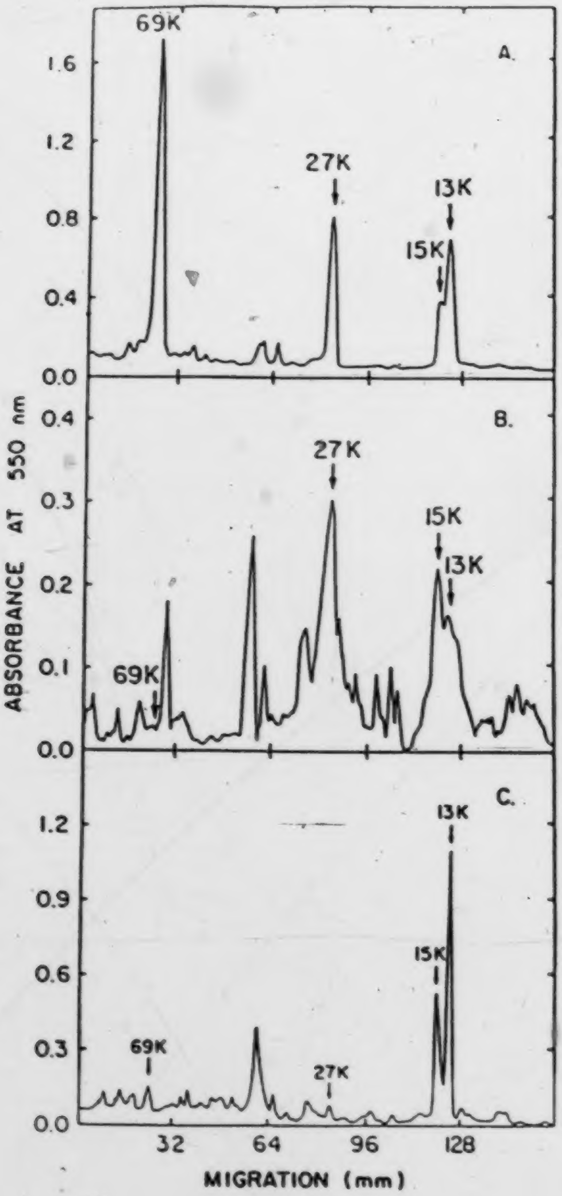

Figure III.2 Densitometric scans of the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns. Densitometric scans of the SDS-PAGE pattern of (A) French press membrane vesicles from HB101/pFRD63); (B) as in (A) but after 30 min chymotrypsin digestion; (C) as in (A) but after 6 M urea wash.

formed a "fuzzy" coating on the vesicle surface and tended to clump the vesicles (Figure III.1d).

In addition to the knob-covered vesicles, we observed an unusual structure in the electron micrographs. This structure appeared to be long tubules covered with a helical array of the knobs (Figure III.1b). The length of these tubes varied greatly, from a few nm to over 1000 nm in length. Attempts to separate the tubules from the vesicles by gel filtration on Biogel A5M or by sucrose gradient sedimentation have been unsuccessful, apparently due to fragmentation of the tubules and to the collapse of the vesicles into tube-like structures.

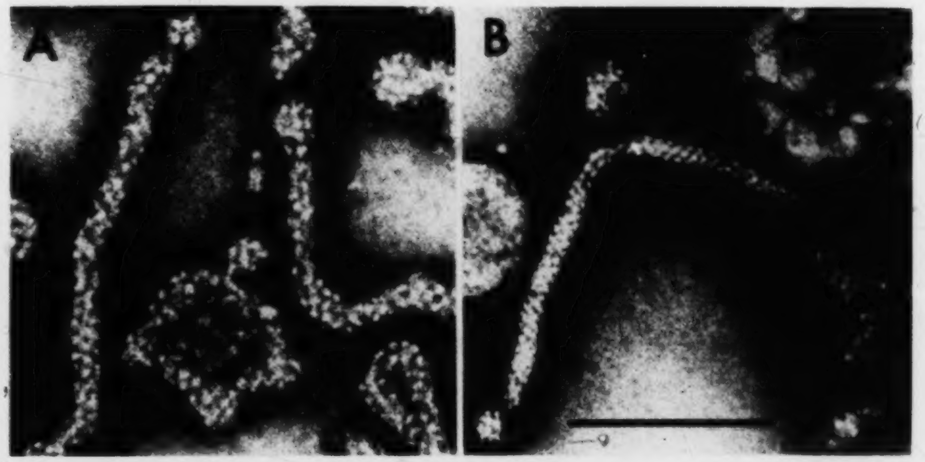

Figure III.3 Electron micrographs of normal and proteolyzed membranes from pFRD63. Electron micrographs of (A) inner membranes from HB101/pFRD63; (B) as in (A) but after chymotrypsin digestion. The bar represents 100nm.

Catalytic Portion of Fumarate Reductase

From the proteinase K digestion experiments it appeared that the presence of the knobs and stalks correlated with the presence of the 69 Kd and 27 Kd subunits. Previous studies (Dickie and Weiner, 1979; Lemire *et al.*, 1982) have shown that these two subunits form the catalytic portion of the enzyme.

To confirm that this structure was indeed the "catalytic head" of the enzyme, two types of experiments were carried out. Treatment of the vesicles with chymotrypsin as described in Methods resulted in removal of the majority of the 69 Kd subunit whereas the 27, 15 and 13 Kd subunits remained membrane-associated as judged by SDS polyacrylamide gel electrophoresis (Figure III.2b). In the electron microscope the chymotrypsin-treated vesicles and tubules presented a more uniform appearance. Knob-like structures could still be seen but these were more distinct and about 20 percent smaller than untreated knobs (3.4 nm versus 4 nm) (compare Figure III.3a and III.3b). It may be that the 69 Kd subunit appears as fuzzy knobs because it is not a tightly packed globular polypeptide. The remnants of the structure seen after chymotrypsin digestion may be the stalks which appear much more uniform and distinct.

Treatment of the vesicles with 6 M urea resulted in 90% removal of the 69 and 27 Kd subunits and small amounts of the 15 Kd subunit (Figure III.2c). These vesicles appeared structureless in the EM (Figure III.4a). The urea wash

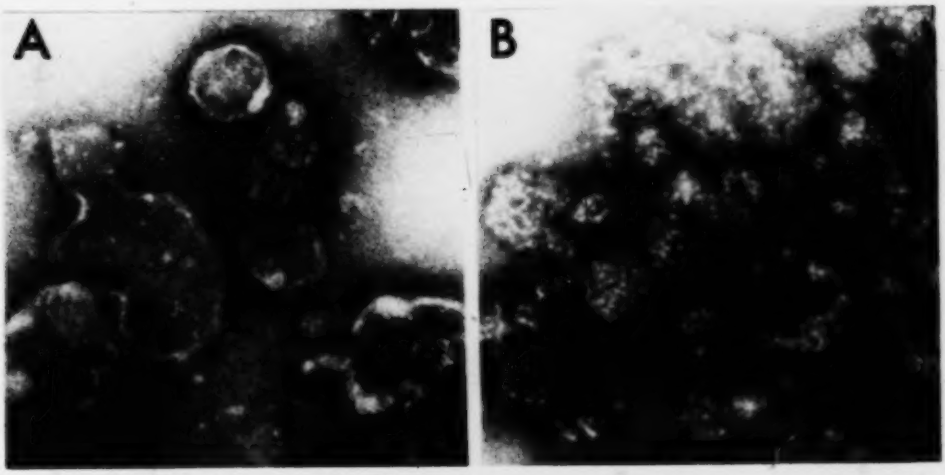

Figure III.4 Electron micrographs of urea stripped and reconstituted membranes. Electron micrographs of (A) 6 M urea washed membranes of HB101/pFRD63; (B) as in (A) but after incubation with HB101/pFRD117 supernatant. The bar represents 100nm.

supernatant was inactive and attempts to regain fumarate reductase activity by dialysis were unsuccessful.

Reconstitution of the Catalytic Portion of Fumarate Reductase with Urea-Stripped Membranes

Reconstitution studies were carried out in order to show that the urea-stripped membranes contained intact anchor polypeptides. Two sources of the catalytic portion of fumarate reductase were used for reconstitution. The first was the crude, soluble, detergent-free form of the enzyme synthesized in strains harboring the plasmid pFRD117. This plasmid codes only for the 69 Kd and 27 Kd subunits and under anaerobic growth conditions produces 10-15% of its protein as fumarate reductase. The second source of enzyme was the two subunit activity purified from the Triton X-100 extract of membranes (of strains harboring the plasmid pFRD63) by hydrophobic exchange chromatography (Lohmeier *et al*., 1981). This enzyme is homogeneous but contains trace amounts of deoxycholate. The results obtained were essentially identical with both preparations.

Incubation of urea-stripped vesicles from HB101/pFRD63 with the catalytic portion resulted in membrane binding of activity and visible reconstitution of the knob-like structure (Figure III.4b). This reconstitution was saturable as shown in Figure III.5 and the binding was specific for vesicles with amplified amounts of anchor polypeptides. Urea-stripped vesicles from HB101/pBR322, which contain only the wild-type level of anchor subunits, did not saturably bind the catalytic heads. The binding was rapid and essentially complete in 10 min. It was independent of temperature between 0° and 37°C, of ionic strength between 0.03 M and 0.2 M NaCl, and of Mg^{++} ion. The presence of DTT was necessary for optimal reconstitution.

We took advantage of several properties of the holoenzyme to distinguish between true reconstitution and surface adsorption. As we have shown previously, the anchor polypeptides modulate several catalytic properties of fumarate reductase (Lemire *et al*., 1982). Thus, the catalytic dimer is very alkaline and thermolabile whereas the holoenzyme is alkaline and thermostable. When the catalytic

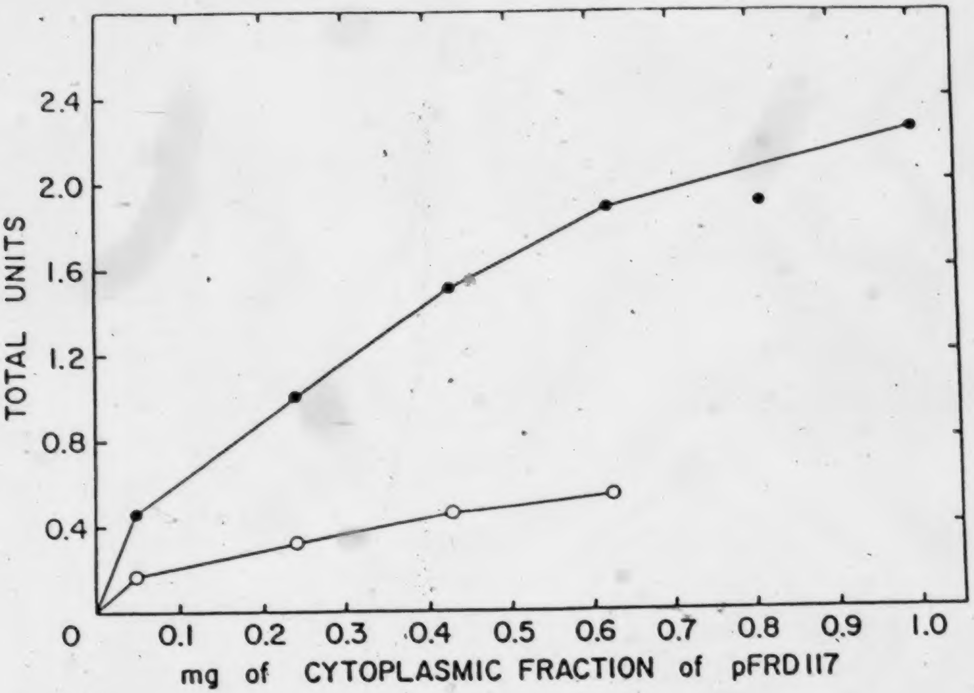

Figure III.5 Reconstitution of the soluble fumarate reductase of HB101/pFRD117 with urea stripped membranes. Reconstitution was carried out as described in Methods. ●-● urea-stripped HB101/pFRD63 membranes; o-o urea-stripped HB101/pBR322 membranes.

portion was reconstituted with anchor polypeptides in the membrane it regained both alkaline stability (Figure III.6) and thermostability (data not shown). The activity of reconstituted membranes incubated at 45°C for 10 minutes decreased by approximately 5% while the activity of the HB101/pFRD117 cytoplasmic fraction decreased by over 90%. The small amount of enzyme bound to HB101/pBR322 membranes did not regain these stability properties (data not shown).

The two subunit form of fumarate reductase requires anions for optimal activity whereas the membrane-bound form

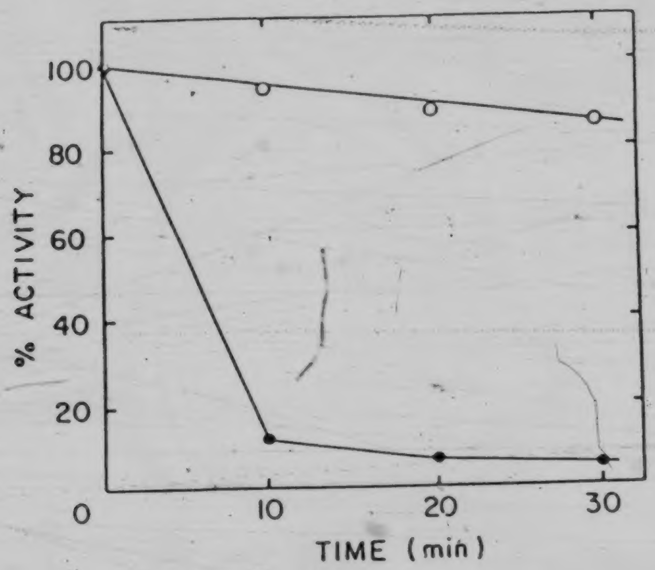

Figure III.6 Thermostability of reconstituted fumarate reductase. Fumarate reductase from the cytoplasm of HB101/pFRD117 (1.8 mg) was reconstituted with urea-stripped French press vesicles (40 μg) of HB101/pFRD63 for 60 min at 0°C and heat lability at 45°C measured as described in Methods. ●-● cytoplasmic fraction; o-o reconstituted membranes.

does not (Robinson and Weiner, 1982). As shown in Table III.1 the reconstituted enzyme is anion independent, as predicted for true reconstitution.

D. DISCUSSION

The results in this paper show that fumarate reductase consists of a membrane-extrinsic domain composed of a catalytic dimer (the 69 Kd and 27 Kd subunits) and a membrane intrinsic or anchor portion composed of the 15 Kd and 13 Kd

Table III.1

Stimulation of fumarate reductase activity by potassium phosphate

Enzyme	Assay A Sp.Ac.	Assay B Sp.Ac.	Ratio B/A
pFRD117 cytoplasmic fraction	14	76	5.4
Purified dimeric fumarate reductase	154	682	4.4
Reconstituted inner membranes	17	21	1.3

Enzyme was assayed in either (A) 25 mM HEPES(*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)(pH 6.8) or (B) 0.2 M potassium phosphate (pH 6.8). Sp.Ac.=specific activity in units per milligram protein.

subunits. The extrinsic portion likely exists as 4 nm diameter knobs attached to the cytoplasmic surface of the membrane by short stalks. It appears that the knobs can be removed by chymotryptic digestion leaving remnants of the structure, presumably the stalks, bound to the membrane. Urea washing of the membranes removes both the 69 Kd and 27 Kd subunits and these vesicles appear structureless in the electron microscope. The anchor subunits are apparently unaffected by the urea wash and can specifically bind fresh catalytic dimer. The anchor subunits not only bind the catalytic dimer to the membrane but also modulate the activity and stability of the catalytic subunits. Our working model for the structure of the enzyme is shown diagrammatically in Figure III.7. In many ways this structure is analogous to the F_0/F_1 ATPase of bacterial and mitochondrial membranes which is composed of an extrinsic F_1 portion and an intrinsic F_0 portion (Fillingame, 1980). The large knob is presumably the 69 Kd and the stalk, the 27 Kd subunit.

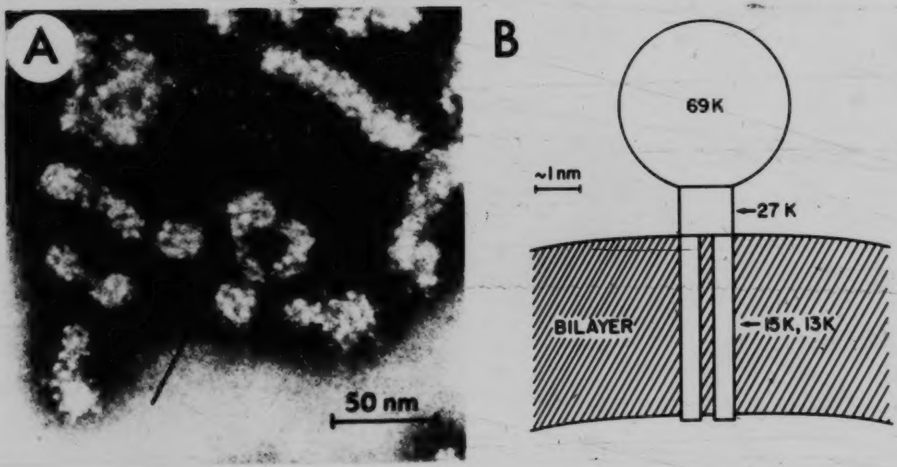

Figure III.7 Model for the structure of fumarate reductase. (A) Electron micrograph of fumarate reductase. The arrows show the knob and stalk aspect of the membrane extrinsic domain of the enzyme. (B) working model of the structure based on the electron micrograph.

The two anchor polypeptides are shown as transmembranal tubes. The actual subunit contacts however, remain to be determined. The transmembranal structure of the hydrophobic anchor polypeptides is based on structure analysis of the sequence (Grundström and Jaurin, 1982). Both proteins consist of three regions composed of very hydrophobic amino acids, each about 23-28 amino acids in length joined by short segments of charged amino acids (Grundström and Jaurin, 1982; Weiner *et al.*, 1984). These hydrophobic regions are long enough to cross the membrane as α-helical structures. The subunit interactions and orientations predicted by this model are presently being probed.

Our ability to reconstitute the catalytic dimer with the anchor portion should prove useful for characterizing fumarate reductase mutants and for identifying the amino acids which form the contact regions. The tubular structures covered with a helical array of the fumarate reductase knobs remain a mystery. They are most numerous in stationary phase cells and may result from vesicle collapse as an artefact of the French Pressure lysis procedure or other manipulations. Attempts to separate vesicles from tubules to examine lipid:protein ratios and subunit composition have so far been unsuccessful.

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IV. A MODEL FOR THE STRUCTURE OF FUMARATE REDUCTASE IN THE CYTOPLASMIC MEMBRANE OF *ESCHERICHIA COLI*

A. INTRODUCTION

Anaerobic growth of *Escherichia coli* on medium containing glycerol (or glycerol-3-phosphate) plus fumarate results in the induction of a very simple electron transport chain which conserves the redox energy of electron flow from glycerol-3-phosphate to fumarate as a proton motive force across the membrane (Harold, 1976). The membrane-associated electron transport chain is composed of only three proteins: an anaerobic glycerol-3-phosphate dehydrogenase, a b type cytochrome and fumarate reductase (Haddock and Jones, 1977). Recently, a combination of genetic, physical, enzymatic and electron microscopic studies have converged to suggest a model for the structure of the terminal electron transfer enzyme of this pathway, fumarate reductase, which may be relevant to mitochondrial succinate dehydrogenase and to other membrane-associated iron-flavo-enzyme complexes.

In this paper, we propose to review the data relevant to the structure of fumarate reductase and to propose a model for the enzyme complex within the cytoplasmic membrane.

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[1] A version of this chapter has been published.
Weiner, J.H., B.D. Lemire, R.W. Jones, W.F. Anderson and D.G. Scraba. 1984. *J. Cell. Biochem.* *24*:207-216.

B. RESULTS AND DISCUSSION

Genetic Approaches

The fumarate reductase operon is located at 93.5 min on the circular *E. coli* map (Bachmann and Low, 1980). Mutants in the structural genes have been isolated (Spencer and Guest, 1973; Lohmeier *et al.*, 1981) and complementation approaches have been used with these mutants to identify a plasmid from the Clarke and Carbon colony bank (Clarke and Carbon, 1976), or from a λ phage -*E. coli* bank (Cole and Guest, 1979), carrying the fumarate reductase operon. The fumarate reductase operon lies on a 4.4 Kb Hind III fragment of *E. coli* DNA and the entire DNA sequence, and thus inferred amino acid sequence, of this fragment has been determined, primarily by S.T. Cole (Cole, 1982; Cole *et al.*, 1982; Grundström and Jaurin, 1982).The fragment codes for 4 polypeptides of 69,000, 27,000, 15,000 and 13,000 daltons which are now termed the *frd*A, *frd*B, *frd*C and *frd*D polypeptides, respectively. Cloning of the Hind III fragment into the multicopy vector pBR322 results in a 20-fold amplification of membrane-associated fumarate reductase activity in strains harboring this plasmid under inducing conditions (Lemire *et al.*, 1982). We have taken advantage of these highly enriched membranes to study the enzyme in its native environment and as a starting material for enzyme isolation.

The Membrane Extrinsic Domain

Our initial isolation of fumarate reductase indicated that the catalytic activity was associated with an $\alpha\beta$ dimer composed of 69,000 and 27,000 dalton subunits (Dickie and Weiner, 1979). This form of the enzyme can be readily isolated in a nearly homogeneous form from normal or amplified membrane fractions of anaerobically grown cells by Triton X-100 solubilization and hydrophobic exchange chromatography on a Phenyl Sepharose column in the presence of deoxycholate (Dickie and Weiner, 1979). The isolated detergent-depleted dimer has been extensively characterized by physical and enzymatic techniques (Robinson and Weiner, 1982). Most relevant to this discussion was the finding that the enzyme-bound only 0.15 gm of Triton X-100 per gm of protein, placing it at the lower limit of membrane-bound proteins (Clarke, 1975). This was unexpected in light of the finding that the enzyme could not be readily removed from the membrane by high or low salt washes, 0.2M EDTA or other procedures known to elute extrinsic proteins (Dickie and Weiner, 1979). Release required complete disruption of the membrane by non-ionic detergent. An additional unexpected property was the finding that the purified dimer required anions for activity and optimal thermal and alkaline stability, unlike the membrane-bound activity (Robinson and Weiner, 1981). As will be shown below this latter property results from removal of the 15,000 and 13,000 dalton membrane-anchor subunits. The

69,000 dalton subunit contains an 8α N-3 histidyl flavin adenine dinucleotide cofactor (FAD covalently linked to a histidine residue in the polypeptide chain) (Weiner and Dickie, 1979). Sequence studies of the 69,000 dalton subunit carried out by Cole resulted in identification of a histidine-containing peptide of identical sequence to the covalent flavin binding domain reported by Singer and Kearney (Singer *et al*., 1973) some years earlier for beef heart mitochondrial succinate dehydrogenase. It is presumed that the flavin plays an essential role in electron transfer within fumarate reductase, as it does in succinate dehydrogenase (Singer *et al*., 1973).

Examination of the sequence of the 69,000 dalton subunit by a Kyte-Doolittle type of analysis (Kyte and Doolittle, 1982) for stretches of hydrophobic amino acids, which might serve as a membrane-binding domain, failed to identify any region of significant hydrophobic character (data not shown).

The 27,000 dalton subunit appears to be the iron-sulfur containing polypeptide. The catalytic dimer (69,000 and 27,000 dalton subunits) has been shown to contain 4 acid labile sulfur atoms and 4 non-heme iron atoms per dimer (Cole *et al*., 1982). The sequence of the 27,000 dalton subunit has an unusual clustering of four cysteine residues with striking homology to the iron-sulfur binding domain of *Peptococcus aerogenes* ferrodoxin (Cole *et al*., 1982) and we presume that this is the iron-sulfur binding site. Although not yet conclusively shown, it appears that a single center of the Fe_4S_4 type is present in the enzyme.

As with the 69,000 dalton subunit, an examination of the hydrophobicity of the 27,000 dalton subunit failed to identify any potential membrane binding domains.

The Membrane Intrinsic Domain

When DNA sequence analysis of the *frd* region of the *E. coli* chromosome was carried out it became clear that two open reading frames corresponding to polypeptides of 15,000 and 13,000 daltons were located downstream from the *frd*B gene (Grundström and Jaurin, 1982). Recently, we showed that these open reading frames code for components of a fumarate reductase complex (Lemire *et al.*, 1982). Two small polypeptides of the expected molecular weights could be visualized by Coomassie blue staining of 10-25% sodium dodecyl sulfate polyacrylamide gradient gels of membranes containing amplified levels of fumarate reductase. We showed that the 15,000 and 13,000 dalton polypeptides were synthesized upon induction of the *frd* operon, were amplified coordinately with the *frd*A and *frd*B polypeptides, and were present in 1:1 mole ratio with the *frd*A and *frd*B polypeptides (Lemire *et al.*, 1982). Most importantly, a holoenzyme containing equimolar amounts of each subunit was solubilized from the membrane by Triton X-100 treatment and the holoenzyme could be purified by sucrose gradient sedimentation (Lemire *et al.*, 1982). In our earlier studies of the enzyme we failed to detect the two small polypeptides because their extremely hydrophobic composition caused them to bind very tightly to the Phenyl Sepharose column. Apparently when we eluted the reductase activity from the column, it eluted not from the resin, but from the hydrophobic polypeptides. These two hydrophobic polypeptides could be recovered from the column by washing with 1% Triton X-100 (Lemire *et al.*, 1982).

The functional role of the two small polypeptides is at least two fold. First they serve as anchor polypeptides for the catalytic dimer. We have shown this in two ways. In one approach a recombinant plasmid, pFRD117, was constructed which carried only the 69,000 and 27,000 dalton sequences in the vector pBR322 (Lemire *et al.*, 1982). Strains harboring this plasmid and induced by anaerobic growth on fumarate produced very high levels of fumarate reductase but all the activity was in the soluble cytoplasmic fraction. This activity behaved as a typical soluble protein and could be purified by conventional techniques without resorting to detergents. In a different approach we prepared inverted membrane vesicles by French press lysis of an induced strain harboring a plasmid carrying the entire operon. When such vesicles were washed with 6M urea the 69,000 and 27,000 dalton subunits were removed, however, the two hydrophobic polypeptides remained membrane-associated. These 'urea-stripped' vesicles can specifically bind fresh catalytic dimers in a saturable fashion (Lemire *et al.*, 1983).

The second function of the hydrophobic polypeptides is to modulate the activity and stability of the catalytic dimer. The catalytically active dimer requires anions for activity and stability whereas the holoenzyme (tetramer), or the membrane-bound activity does not (Lemire *et al.*, 1982; Robinson and Weiner, 1981).

Secondary Structure Predictions for the Anchor Polypeptides

The anchor polypeptides have been shown to lack cleaved leader sequences (Grundström and Jaurin, 1982), thus the inferred amino acid sequences obtained from DNA sequence analysis correspond to the functional proteins. We have examined these sequences for relative hydrophobicity and hydrophilicity according to the method of Kyte and Doolittle (Kyte and Doolittle, 1982). As can be seen in Figure IV.1 each of the anchor polypeptides has three relatively long stretches of hydrophobic amino acids separated by relatively short stretches of charged and polar residues. By analogy to other membrane-bound proteins (Engelman *et al.*, 1980) it seemed likely that the hydrophobic stretches were in an α-helical configuration. To further test this idea, the *frd*C and *frd*D amino acid sequences were compared with each other and with the amino acid sequence of bacteriorhodopsin. The procedure used will be reported elsewhere (Anderson, in preparation). Briefly the comparison of the sequences is by the method of Fitch (Fitch, 1966) except that the scores relating individual amino acids were assigned on the basis

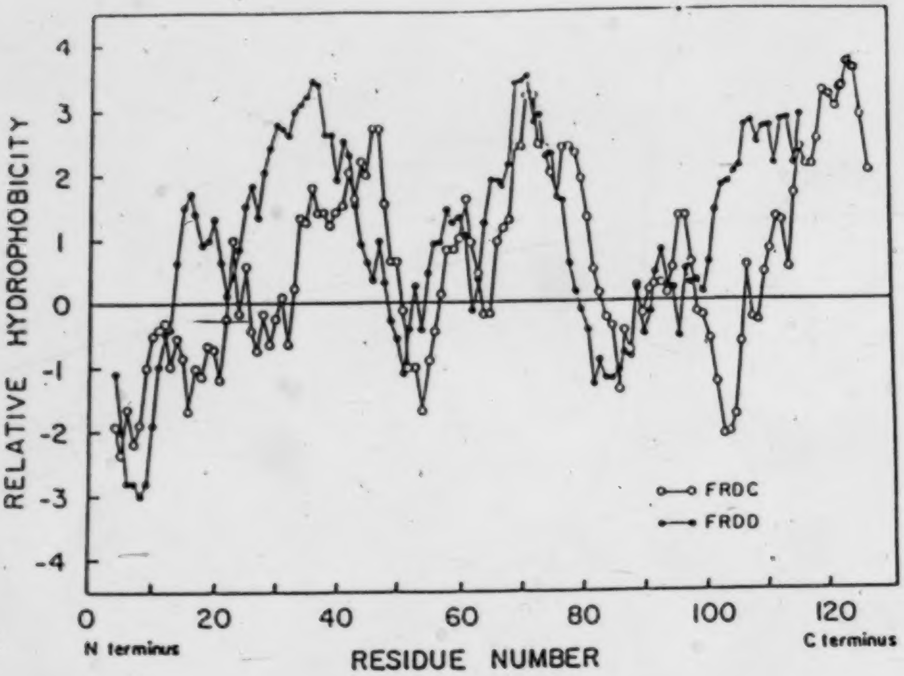

Figure IV.1 Hydrophobicity plots. Plot of hydrophobicity versus position in the amino acid sequence for *frd*C (o-o) and *frd*D (•-•). The hydrophobicity values are averages of the Kyte-Doolittle parameters over four consecutive residues (Kyte and Doolittle, 1982).

of structural similarities of the amino acids. Structural similarities used rather than the genetic code (Fitch, 1966) or observed amino acid substitutions in homologous sequences (McLachlan, 1971) because the aim is to detect regions which potentially have similar structures and are not necessarily related through divergent evolution. The score for replacing one amino acid with another is derived from the difference between the two amino acids in terms of hydrophobicity (Kyte and Doolittle, 1982; Manavalan and Ponnuswamy, 1978), secondary structure preferences

(Levitt, 1978), polarity and size. The scores used for the replacement of any amino acid by any other are given in Table IV.1. This table of individual scores is used to calculate the score for each span of residues for every alignment and the matrix of scores obtained is then displayed as in Figures IV.2 and IV.4.

When this analysis was carried out on the *frd*C polypeptide versus bacteriorhodopsin, the matrix (Figure IV.2) does not show the strong diagonal features that occur with homologous amino acid sequences, but rather they indicate regions of the sequences with similar structural preferences (the darker patches indicated by the arrows on the abcissa of Figure IV.2) Similar results were obtained when the *frd*D protein was compared with bacteriorhodopsin. The regions of similarity correspond to segments of the bacteriorhodopsin structure known to be α-helical (Engelman *et al.*, 1980) and to be embedded in the lipid bilayer. It thus seems likely that the anchor polypeptides of fumarate reductase have similar structural features and are embedded in the bilayer. The length of the hydrophobic segments correspond to lengths of about 24 ± 4 amino acid residues; such a stretch of polypeptide would be long enough to traverse the hydrophobic core of a lipid bilayer of 40Å. Secondary structure calculations also predict reverse turns in the non-hydrophobic segments. In Figure IV.3a and IV.3b we propose a model for the secondary structure of the *frd*C and *frd*D polypeptides within the lipid bilayer which incorporates these

Table IV.1

Amino acid similarities

	ALA (A)	ARG (R)	ASN (N)	ASP (D)	CYS (C)	GLN (Q)	GLU (E)	GLY (G)	HIS (H)	ILE (I)	LEU (L)	LYS (K)	MET (M)	PHE (F)	PRO (P)	SER (S)	THR (T)	TRP (W)	TYR (Y)	VAL (V)
ALA	1.00	0.11	-0.25	-0.25	0.21	-0.09	0.10	-0.07	0.17	-0.20	0.21	0.02	0.23	0.12	-0.49	-0.01	0.09	-0.12	0.12	0.21
ARG	-0.11	1.00	0.19	-0.01	-0.29	0.28	0.12	-0.36	0.56	-0.67	-0.45	0.61	-0.24	-0.15	-0.48	0.15	0.29	-0.02	0.08	-0.70
ASN	-0.25	0.19	1.00	0.31	-0.35	0.48	0.11	-0.15	0.04	-0.98	-0.71	0.23	-0.46	-0.52	-0.17	0.50	0.34	-0.33	-0.13	-0.98
ASP	-0.25	-0.01	0.31	1.00	-0.51	0.12	0.29	-0.12	-0.15	-1.12	-0.81	0.13	-0.64	-0.65	-0.03	0.31	0.11	-0.53	-0.36	-1.13
CYS	0.21	-0.29	-0.35	-0.51	1.00	-0.20	-0.10	-0.28	-0.04	-0.02	0.38	-0.28	0.41	0.11	-0.72	-0.29	-0.19	0.07	-0.04	-0.02
GLN	-0.09	0.28	0.48	0.12	-0.20	1.00	0.34	-0.42	0.30	-0.83	-0.49	0.34	-0.14	-0.33	-0.53	0.20	0.22	-0.17	-0.14	-0.85
GLU	0.10	0.12	0.11	0.29	-0.10	0.34	1.00	-0.34	0.24	-0.69	-0.31	0.24	-0.06	-0.20	-0.50	0.07	0.09	-0.14	-0.16	-0.72
GLY	-0.07	-0.36	-0.15	-0.12	-0.28	-0.42	-0.34	1.00	-0.39	-0.70	-0.52	-0.28	-0.58	-0.47	0.34	0.23	0.04	-0.50	-0.19	-0.66
HIS	0.17	0.56	0.04	-0.15	-0.04	0.30	0.24	-0.39	1.00	-0.35	-0.10	0.43	0.14	0.18	-0.64	0.07	0.31	0.18	0.18	-0.39
ILE	-0.20	-0.67	-0.98	-1.12	-0.02	-0.83	-0.69	-0.70	-0.35	1.00	0.38	-0.80	0.09	0.36	-1.23	-0.78	-0.53	-0.01	-0.09	0.86
LEU	0.21	-0.45	-0.71	-0.81	0.38	-0.49	-0.31	-0.52	-0.10	0.38	1.00	-0.47	0.51	0.43	-0.98	-0.57	-0.39	0.13	-0.09	0.31
LYS	0.02	0.61	0.23	0.13	-0.28	0.34	0.24	-0.28	0.43	-0.80	-0.47	1.00	-0.25	-0.30	-0.37	0.21	0.21	-0.22	-0.16	-0.82
MET	0.23	-0.24	-0.46	-0.64	0.41	-0.14	-0.06	-0.58	0.14	0.09	0.51	-0.25	1.00	0.34	-1.01	-0.42	-0.23	0.18	-0.07	0.03
PHE	0.12	-0.15	-0.52	-0.65	0.11	-0.33	-0.20	-0.47	0.18	0.36	0.43	-0.30	0.34	1.00	-0.88	-0.37	-0.10	0.48	0.32	0.29
PRO	-0.49	-0.48	-0.17	-0.03	-0.72	-0.53	-0.50	0.34	-0.64	-1.23	-0.98	-0.37	-1.01	-0.88	1.00	0.07	-0.23	-0.78	-0.50	-1.21
SER	-0.01	0.15	0.50	0.31	-0.29	0.20	0.07	0.23	0.07	-0.78	-0.57	0.21	-0.42	-0.37	0.07	1.00	0.61	-0.33	-0.02	-0.77
THR	0.09	0.29	0.34	0.11	-0.19	0.22	0.09	0.04	0.31	-0.53	-0.39	0.21	-0.23	-0.10	-0.23	0.61	1.00	-0.11	0.20	-0.51
TRP	-0.12	-0.02	-0.33	-0.53	0.07	-0.17	-0.14	-0.50	0.18	-0.01	0.13	-0.22	0.18	0.48	-0.78	-0.33	-0.11	1.00	0.50	-0.08
TYR	-0.12	0.08	-0.13	-0.36	-0.04	-0.14	-0.16	-0.19	0.18	-0.09	-0.09	-0.16	-0.07	0.32	-0.50	-0.02	0.20	0.50	1.00	-0.11
VAL	-0.21	-0.70	-0.98	-1.13	-0.02	-0.85	-0.72	-0.66	-0.39	0.86	0.31	-0.82	0.03	0.29	-1.21	-0.77	-0.51	-0.08	-0.11	1.00

1.00 corresponds to perfect similarity

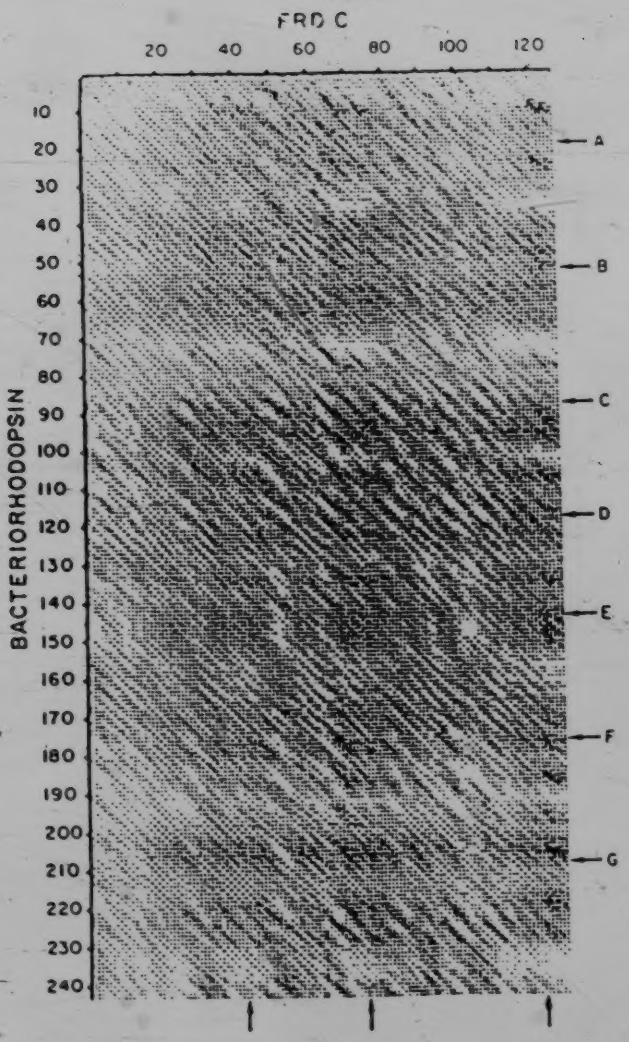

Figure IV.2 Similarity of bacteriorhodopsin and *frd*C. Matrix relating the degree of similarity of the bacteriorhodopsin and the *frd*C amino acid sequences. Each point in the matrix is the result of the comparison of a 10 residue segment of bacteriorhodopsin with a 10 residue segment of *frd*C. The similarity is scored according to the structural attributes (secondary structure preferences, hydrophobicity and hydrophilicity, size and charge) of each of the aligned amino acids (W.A., unpublished). The scores of all possible ten residue comparisons are then fit by a standard gaussian and the matrix plotted such that the better the score (in standard deviations from the mean), the larger the black dot. The positions of the proposed seven transmembrane α-helices in bacteriorhodopsin (Engelman *et al*., 1980) are indicated by the arrows A-G. The regions of the *frd*C polypeptide indicated by the arrows are the proposed transmembranal α-helices.

predictions. Each polypeptide weaves through the membrane three times and although they bear little sequence homology, they are strikingly similar in overall structure when analyzed by the comparative approach described above (Figure IV.4). In this model very few charges are buried within the lipid bilayer. The *frd*C protein has a lysine and glutamate between residues 51 and 55 which will make a salt bridge. A lone glutamate is found in the *frd*C hydrophobic domain and a lone arginine in the bilayer spanning portions of the *frd*D protein. These residues may have important functional roles. Several of the helices have proline residues that will probably introduce bends of approximately 20° in the helices, but may not distort the overall helical conformation.

The overall orientation of the protein is unknown, however the orientation shown in Figure IV.3 seems most likely as it provides segments of the anchor polypeptides to interact with the catalytic subunits which are known to be on the cytoplasmic surface and aligns the polypeptide with the membrane potential (internal negative). This structure also suggests a mechanism for anchor assembly within the bilayer. The positive charges at the N-terminus may allow the growing polypeptide to bind to the membrane by ionic interaction in a fashion analogous to that proposed for signal sequences (Inouye and Halegoua, 1978). Subsequently, the first two α-helical segments may enter the membrane as a helical hairpin (Inouye *et al*., 1982) and the third segment

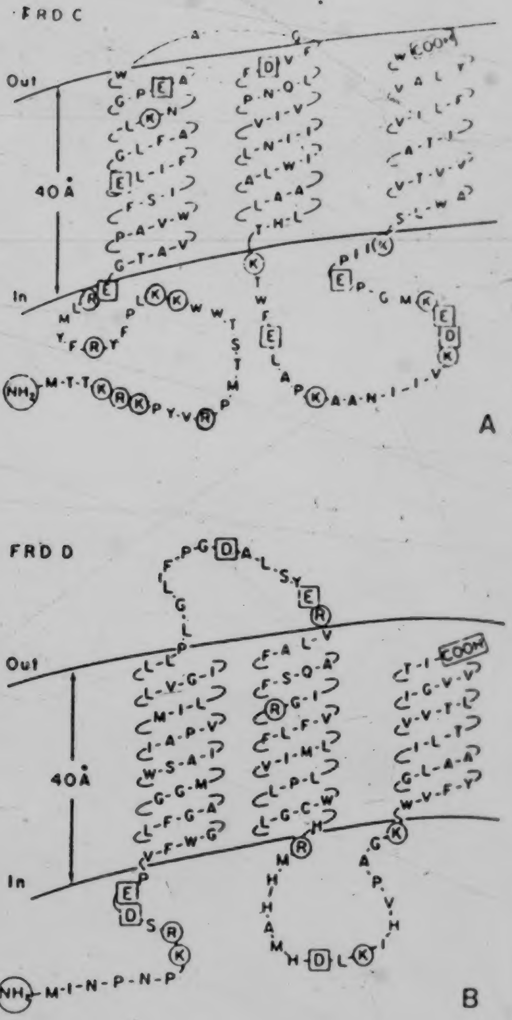
FRD C
Out
40 Å
In
NH2
COOH
A
FRD D
Out
40 Å
In
NH2
COOH
B

Figure IV.3 Membrane organization of the anchor polypeptides. A model for the organization of the *frd*C protein (A) and the *frd*D protein (B) with respect to the lipid bilayer. Amino acids with positively charged side chains are shown as circles and amino acids with negatively charged side chains are shown as squares.

partition into the membrane once polypeptide synthesis is complete and the protein is released from the ribosome.

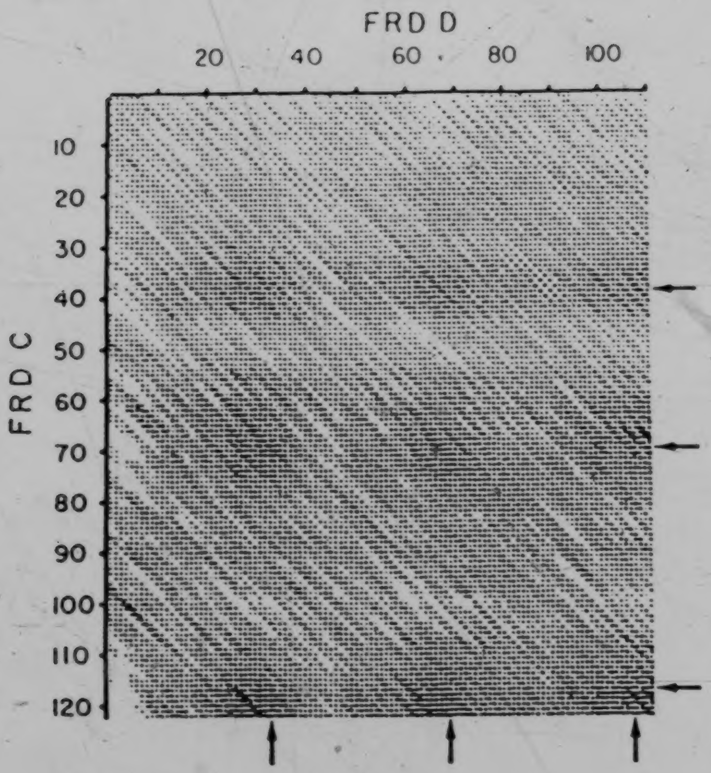

Figure IV.4 Similarity matrix of the *frd*C and *frd*D polypeptides. Note that the regions of higher than average similarity correspond to those regions of *frd*C which also were most similar to the transmembrane α-helices of bacteriorhodopsin and that these regions of similarity between *frd*C and *frd*D coincide with the hydrophobic segments of the polypeptides (Figure IV.2).

Electron Microscopic Studies

When negatively stained preparations of the membranes highly enriched in fumarate reductase were examined in the electron microscope a knob and stalk type of structure was observed on the cytoplasmic surface which correlated with fumarate reductase expression (Figure IV.5). We have shown that these extramembranal structures bind anti-fumarate reductase γ-globulin and can be removed from the membranes

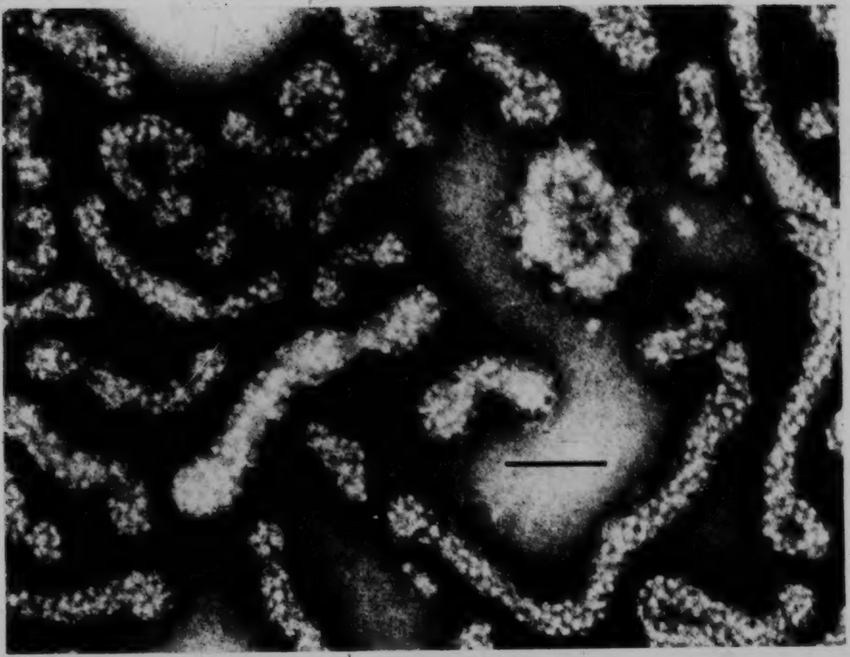

Figure IV.5 Electron micrograph. Electron micrograph of inner membrane vesicles prepared by French press lysis of anaerobically grown *E. coli* HB101 carrying a plasmid with the frd operon (Lemire *et al.*, 1982). The bar represents 50nm.

by the 6M urea wash (Lemire *et al.*, 1983). As described above the wash contained the 69,000 and 27,000 dalton subunits whereas the 15,000 and 13,000 dalton subunits remained associated with the membrane. Reconstitution studies have shown that the anchor polypeptides remain functional and can rebind fresh catalytic dimers in a saturable fashion (Lemire *et al.*, 1983).

In addition to the typical inverted membrane vesicles expected in a French Pressure cell lysis of bacteria, we observed novel tubular structures covered with a helical arrangement of the knobs and stalks (Figure IV.5). These tubules are not an artifact of the lysis procedure and can be observed by thin section electron microscopy in intact bacteria with greatly amplified levels of fumarate reductase. The tubules appear to aggregate into bundles which can extend the entire length of the bacterium. The isolation and biochemical characterization of these tubules will be reported elsewhere (Weiner *et al.*, 1984).

A Model for the Structure of the Holoenzyme

The information summarized in the preceding sections can be combined to generate the working model for fumarate reductase which is shown in Figure IV.6. The enzyme is seen to be composed of a membrane extrinsic domain composed of 69,000 and 27,000 dalton subunits forming the stalk and knobs seen in electron micrographs and a membrane intrinsic domain composed of the 15,000 and 13,000 dalton anchor subunits which we believe to be transmembranal proteins.

The organization of this enzyme complex resembles the F_0/F_1 ATPase in having an extrinsic stalk and knob structure and a transmembranal domain (Fillingame, 1980). In particular, the membrane intrinsic domain resembles similar domains in bacteriorhodopsin (Engelman *et al.*, 1980), the F_0 portion of ATPase (Tzagaloff *et al.*, 1979) and the lac carrier (Goldkorn *et al.*, 1981), three proteins known to be involved in proton translocation. The possibility that fumarate reductase acts as a proton pump is presently under investigation.

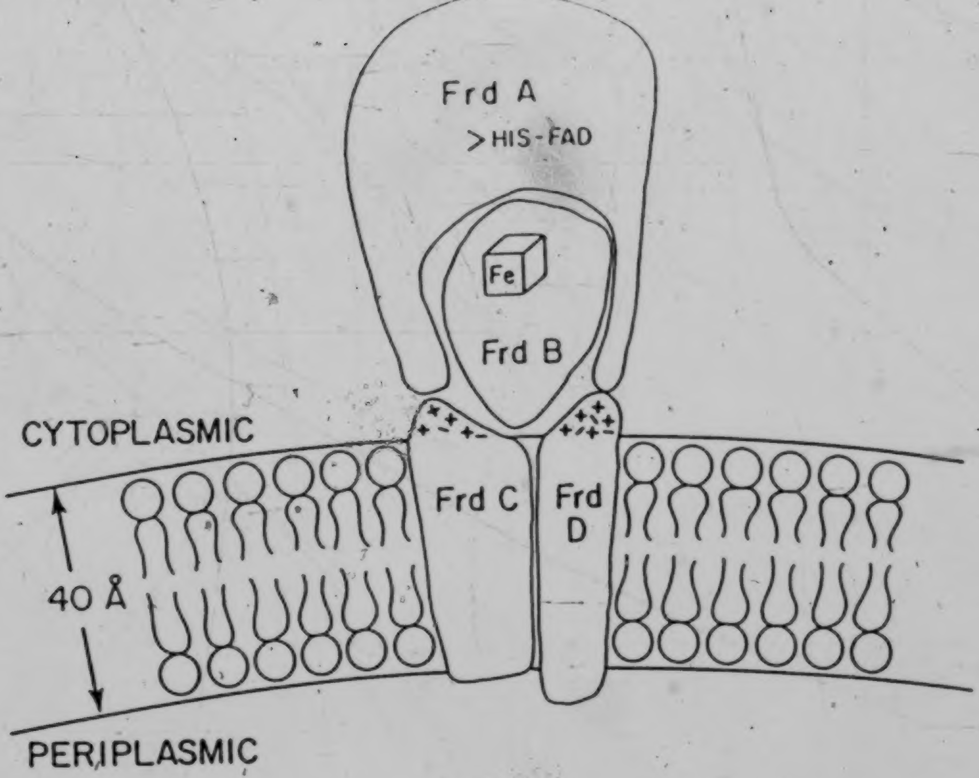

Figure IV.6 A model for the fumarate reductase enzyme complex within the lipid bilayer.

We have found that the 27,000 dalton subunit is partially inaccessible to labeling by phenylglyoxal, iodosulfanilic acid and lactoperoxidase catalyzed iodination (Lemire and Weiner, unpublished results) as well as to chymotrypsin catalyzed proteolysis (Lemire *et al.*, 1983). These results lead us to propose that the 69,000 dalton subunit envelopes most of the 27,000 dalton subunit. Further, crosslinking studies with dithiobis(succinimidylpropionate), show that the 69,000 and 27,000 dalton subunits interact and that the 15,000 dalton subunit can crosslink to the 69,000 dalton subunit, indicating that these two subunits are in close proximity (Robinson and Weiner, 1982; Robinson, 1982). The 15,000 and 13,000 daltons subunits can be crosslinked by dithiobis(succinimidylpropionate) only from the inside surface as would be predicted from examination of Figure IV.3 (all the lysines are on the inside). Further studies of subunit interactions and the possible association of other components of the electron transport chain with fumarate reductase are underway.

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V. OVERPRODUCTION OF FUMARATE REDUCTASE IN *Escherichia coli* INDUCES A NOVEL INTRACELLULAR LIPID-PROTEIN ORGANELLE[1]

A. INTRODUCTION

Anaerobic growth of *E. coli* in the presence of fumarate induces a very simple electron transport chain with fumarate reductase as the terminal electron transfer enzyme (Haddock and Jones, 1977). We have purified and extensively characterized this membrane-bound enzyme and have shown that it is composed of four non-identical subunits in equimolar amounts (Lemire *et al.*, 1982; Lemire *et al.*, 1983). A large 69,000 dalton subunit coded by the *frd*A gene forms part of the catalytic domain and carries the essential covalent flavin cofactor (Weiner and Dickie, 1979). The *frd*B gene codes for a 27,000 dalton subunit which plays a role in non-heme iron binding and catalytic activity (Cole *et al.*, 1982). Two small subunits of 15,000 and 13,000 daltons coded by the *frd*C and *frd*D genes, respectively, are integral membrane proteins which serve to anchor the catalytic subunits to the membrane (Lemire *et al.*, 1982; Weiner *et al.*, 1983). Several groups have cloned the *frd* operon into the plasmid vector pBR322 (Lemire *et al.*, 1982; Cole and Guest, 1980; Grundström and Jaurin, 1982; Lohmeier *et al.*, 1981). We have shown that strains harboring a recombinant plasmid overproduce fumarate reductase 30-40 fold (Lemire

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[1] A version of this chapter has been published. Weiner, J.H., B.D. Lemire, M.L. Elmes, R.D. Bradley and D.G. Scraba. 1984. *J. Bacteriol.* 158:590-596.

et al., 1982; Lemire *et al.*, 1983). Under these growth conditions the enzyme accounts for more than 50% of the inner membrane protein. In a recent paper (Lemire *et al.*, 1983) we examined these enriched membranes by negative-staining electron microscopic techniques and showed that everted membrane vesicles were covered with 40 Å knobs made up of the 69,000 and 27,000 dalton subunits of fumarate reductase. We proposed a model in which the enzyme was composed of a membrane extrinsic catalytic domain (the 69,000 and 27,000 polypeptides) and a membrane intrinsic anchor domain (the 15,000 and 13,000 polypeptides). During the course of the work on the everted membrane preparations we observed not only typical membrane vesicles, but also long tubular structures composed of an array of fumarate reductase. In this paper, we show that these tubules are not an artefact of vesicle preparation but a new lipid-protein structure induced by overproduction of fumarate reductase. We have also examined the lipid and protein composition of the membranes and we have shown that amplification of fumarate reductase does not reduce the levels of other membrane-bound activities.

B. METHODS

Strains and plasmids

E. coli HB101 is F^-, *hsdR*, *hsdM*, *pro*, *leu*, *gal*, *lac*, *thi*, *recA*, *rpsL*. The plasmids pBR322 and pFRD63 have previously been described (Lemire *et al.*, 1982).

Preparation of everted envelopes, inner membranes and cytoplasmic fractions

Cells were grown anaerobically on glycerol-fumarate medium to late stationary phase (Spencer and Guest, 1974) in medium containing 100 μg/ml ampicillin. Everted envelopes were prepared by French Pressure cell lysis (Aminco Instruments, Rockville, Md.) followed by centrifugation at 150,000 xg for 90 min as previously described (Lemire *et al.*, 1982) to separate the supernatant containing the cytoplasmic fraction from the pellet containing the everted envelopes. For some experiments purified inner membranes were prepared by the procedure of Yamato *et al.* (Yamato *et al.*, 1978), except that the dialysis step and final wash were omitted.

Enzyme Assays

Fumarate reductase was assayed by following the oxidation of reduced benzyl viologen as previously described (Dickie and Weiner, 1979). One unit of activity corresponds to 1 μmole of benzyl viologen oxidized per minute.

D(-)lactate dehydrogenase and anaerobic glycerol-3-phosphate dehydrogenase were assayed by following the substrate dependent reduction of MTT as described by Schryvers and Weiner (Schryvers and Weiner, 1981). The glycerol-3-phosphate dehydrogenase assays were carried out in the presence of 10 μM FAD and 1 mM FMN. One unit corresponds to 1 μmole of MTT reduced per minute.

NADH dehydrogenase was assayed by following the NADH dependent reduction of DCPIP as described by Thomson and Shapiro (Thomson and Shapiro, 1981). One unit corresponds to one μmole of DCPIP reduced per minute.

NADH oxidase was measured by directly following the oxidation of NADH at 340 nm (Thomson and Shapiro, 1981). One unit corresponds to one μmole NADH oxidized per minute.

ATPase was assayed by measuring the release of inorganic phosphate as described by Owen *et al*. (Owen *et al*., 1982). One unit corresponds to 1 μmole of P_i released per minute.

Protein determination

Protein was estimated by the Lowry method (Lowry *et al*., 1951) and the Bradford method (Bradford, 1976) using Bio-Rad protein standard (Bio-Rad Laboratories, Richmond, Ca.).

Extraction of membrane lipids

The total lipids were quantitatively extracted from purified cytoplasmic membranes prepared from HB101/pBR322 and HB101/pFRD63 by a modification of the method of Bligh and Dyer (Bligh and Dyer, 1959). A 0.2 ml aliquot of inner membranes was diluted with 8 ml α-buffer (0.05 M Tris-HCl, pH 7.8, 0.15 M NaCl, 0.01 M $MgSO_4$) into a 200 ml screw capped bottle. Ten ml methanol was added, the suspension shaken and the sample incubated at 70°C for 60 min. After cooling, the solution was mixed well with 40 ml of chloroform, 20 ml of water, and 40 ml of chloroform in that order. The solution was transferred to a separatory funnel and the lower chloroform phase was filtered through chloroform-wetted filter paper.

The chloroform solution was passed through a chromatographic column prepared from 10 g silicic acid in chloroform. The column was washed with 3 column volumes of chloroform and eluted with 3 column volumes of methanol. The eluant contained the total membrane lipid essentially free of non-lipid contaminants.

Analysis of fatty acids by gas-liquid chromatography

Methyl esters of fatty acids were prepared from lipids by transesterification according to the method of Gander *et al.*(Gander *et al.*, 1962). Solvent was evaporated under a stream of nitrogen at 24°C in a screw capped test tube. Five ml methanol and 0.5 ml concentrated sulfuric acid were added and the tube was incubated at 70°C for 60 min. After cooling, the contents of the tube were mixed with 10 ml water, and the methyl esters were extracted with 2 ml hexane. The extracted hexane solution was dried with anhydrous sodium sulfite and dried down under nitrogen. The methyl esters were redissolved in a known volume of hexane and injected into a Hewlett-Packard model 5700A equipped with a model 3390A electronic integrator.

The ~~gas-li~~quid chromatography was carried out as described by Saito and McElhaney (Saito and McElhaney, 1977). Fatty acids were identified on the basis of retention times as compared with known standards. The proportion of each fatty acid present in each sample was calculated from the area under each peak, as determined by the electronic integrator.

Quantitation of membrane phospholipids

Cultures of HB101/pBR322 and HB101/pFRD63 were labelled by the addition of 1 µCi/ml inorganic ^{32}P to the growth medium. Lipids were extracted as described above. The individual phospholipids were separated by two dimensional thin-layer chromatography on glass plates coated with Silica Gel H in solvent systems of [i] chloroform:methanol:7N NH_4OH (60:35:5 v/v) and [ii] chloroform:methanol:acetic acid (65:25:8 v/v) (Ames, 1968). The positions of the lipids were visualized by exposure to iodine vapour, the silica gel scraped from the plates and the relative amounts of each lipid was determined by liquid-scintillation counting in ACS (Amersham, Chicago, Ill.). The lipid components were tentatively identified by comparing R_f values with those previously reported by Ames (Ames, 1968).

Inorganic Phosphate determination

Inorganic phosphate was determined according to the method of Ernster *et al.* (Ernster *et al.*, 1950) using phosphate standards (BDH Chemicals, Edmonton, Alta.).

Thin section electron microscopy

HB101/pFRD63 and control cells were collected by centrifugation, washed with 0.2 M sodium phosphate (pH 7.2) and fixed with 2% glutaraldehyde. They were then washed with phosphate-buffered sucrose, post-fixed in 2% OsO_4 (in 0.2 M sodium phosphate, 1.7% sucrose), washed with sucrose free buffer, dehydrated in ethanol and embedded in Epon 812. Sections of 60-120 nm thickness were stained with lead citrate and saturated uranyl acetate and mounted on grids for electron microscopy.

In situ lysis and negative staining electron microscopy

HB101/pFRD63 was grown anaerobically to mid-log phase as described above. Cells were harvested and washed once with cold 10 mM Tris-HCl, pH 7.5 and resuspended at 24°C in 33 mM Tris-HCl, pH 7.5 containing 20% sucrose. EDTA was added to 5 mM followed by lysozyme to 0.1 mg/ml. The suspension was incubated at 37°C for 30 min with occasional stirring and then adsorbed to hydrophilic carbon films, washed twice with sucrose free buffer and negatively stained with 1% sodium phosphotungstate, pH 7.0. Photographs were obtained in a Phillips EM300 electron microscope operated at

80 kV.

C. RESULTS

Amplification of fumarate reductase does not reduce the levels of other membrane-bound enzymes

Our observation that fumarate reductase can account for more than 50% of the cytoplasmic membrane protein led us to examine the effect of amplification in several membrane-bound enzymes. We quantitated the activities of four enzymes, as well as the NADH oxidase electron transfer chain, in envelopes isolated from cells with normal and highly amplified levels of fumarate reductase. The results in Table V.1 show that the total amount of activity, per fixed weight of cells, of D(-) lactate dehydrogenase and glycerol-3-phosphate dehydrogenase remains constant while the activities of NADH dehydrogenase and ATPase decrease about 30% under conditions where the amount of fumarate reductase increases 20-fold. The total amount of membrane-associated protein more than doubles resulting in decreased specific activities for the enzymes measured. Interestingly, NADH oxidase activity, which is present in anaerobically grown *E. coli* (Haddock and Jones, 1977), was totally absent from cell envelopes containing amplified levels of fumarate reductase although NADH dehydrogenase activity was reduced only 30%.

Table V.1

Enzyme activities of cell envelopes containing normal and amplified levels of fumarate reductase

		HB101 pBR322		HB101 pFRD63	
		u/ml	Sp.Ac.	u/ml	Sp.Ac.
Fumarate reductase	cytoplasm	128	0.76	659	4.8
	membrane	321	14.9	6,696	118
D(-)Lactate dehydrogenase	cytoplasm	3.15	0.019	1.19	0.009
	membrane	6.85	0.32	6.67	0.118
G3P dehydrogenase	cytoplasm	29.0	0.173	8.2	0.060
	membrane	9.65	0.45	9.5	0.17
NADH dehydrogenase	cytoplasm	84.6	0.50	123	0.89
	membrane	2.6	0.17	1.73	0.031
ATPase	cytoplasm	1.17	0.14	0.59	0.14
	membrane	1.24	0.29	0.90	0.19
NADH oxidase	membrane	5.90	0.27	<0.1	<0.001

One unit of specific activity (Sp.Ac.) equals one micromole of substrate converted per minute per milligram of protein.

Lipid:protein ratio of fumarate reductase overproducing cells

The results in Table V.2 show that purified cytoplasmic membranes, isolated by the Yamato procedure (Yamato *et al.*, 1978), from cells overproducing fumarate reductase have over double the protein content of membranes produced from normal cells. All of the extra protein is fumarate reductase. This can be calculated by dividing the total activity (8300 units/ml) by the specific activity of purified fumarate reductase holoenzyme (420 units/mg) (Lemire *et al.*, 1982). This calculation indicates that 19.8 mg of fumarate reductase are present, in good agreement with the increase in protein (20.4 mg).

The phospholipid content, quantitated by lipid phosphate as described in Methods, also doubled in the overproducing cells resulting in a relatively constant lipid:protein ratio. The lipid:protein ratio of 0.4 is typical of *E. coli* inner membranes with 60% of the weight being protein (Ingraham *et al.*, 1983). These results indicate that the cells are able to adjust to the overproduction of fumarate reductase by increasing the total amount of inner membrane material.

Phospholipid composition of overproducing cells

The major phospholipids of the *E. coli* inner membrane, phosphatidylethanolamine and phosphatidylglycerol are unaltered by the overproduction of fumarate reductase

Table V.2

Lipid/protein ratios of membranes containing normal and amplified levels of fumarate reductase

Strain	Protein (mg/ml)	Fumarate reductase (U/ml)	Phospholipid (mg/ml)	Lipid/ protein ratio
HB101(pFRD63)	38.6	8,300	14.2	0.367
HB101(pBR322)	18.2	330	7.3	0.401

Phospholipid determined by lipid phosphate. U=units of activity.

(Table V.3) and are both present at normal levels in anaerobically grown cells (Ames, 1968). Interestingly, in the anaerobically grown HB101/pBR322 we could not detect any cardiolipin but we did observe a significant amount of a phospholipid identified as acyl phosphatidylglycerol (Olsen and Ballou, 1971). In HB101/pFRD63, grown under identical conditions, the acyl phosphatidylglycerol component was absent and a large amount of cardiolipin was observed. In

Table V.3

Phospholipid composition of membranes containing normal and amplified levels of fumarate reductase

	% of total lipid in	
Phospholipid	HB101(pBR322)	HB101(pFRD63)
Phosphatidyl ethanolamine	64	61
Phosphatidyl glycerol	27	24
Cardiolipin	ND	15
Acyl phosphatidyl glycerol	9	ND

ND= not detectable

aerobically grown cells cardiolipin is only 5% of the membrane phospholipid (Ingraham *et al.*, 1983) and it may be that the cell is adapting to the overproduction of fumarate reductase by increasing the synthesis of cardiolipin.

Fatty acid composition of fumarate reductase overproducing cells

The fatty acid compositions of membranes containing normal and amplified levels of fumarate reductase are compared in Table V.4. The amplified inner membranes show decreases in the saturated fatty acids tentatively identified as myristate and stearate as well as in the 19:0 cyclopropane. Significant increases are observed in the unsaturated fatty acids tentatively identified as

Table V.4

Fatty acid content of anaerobically grown *E. coli*

Fatty acid	% (wt/wt) of fatty acid in	
	HB101(pBR322)	HB101(pFRD63)
$C_{14:0}$	27.9	17.2
$C_{15:0}$ cyclopropane	1.3	0.8
$C_{16:0}$	38.4	40.1
$C_{16:1}$	3.28	9.97
$C_{17:0}$ cyclopropane	15.0	22.3
$C_{18:0}$	0.98	ND
$C_{18:1}$	1.86	3.6
$C_{19:0}$ cyclopropane	11.1	5.9

ND= not detectable

palmitoleic and oleic as well as the 17:0 cyclopropane. Overall these changes would be expected to lower the phase transition temperature and have a fluidizing effect on the membranes (Jain and Wagner, 1980).

Thin section electron microscopy

The doubling of both membrane protein and lipid per fixed weight of cells indicates that the total amount of membrane in the amplified cells must double. To determine where this extra membrane was localized we examined thin sections of normal and amplified cells in the electron microscope. Figure V.1 shows (A) longitudinal and (B) lateral cross sections of amplified cells containing tubular structures running in a primarily longitudinal direction throughout the cells. In lateral cross section, the cell appears packed with these structures which are composed of an electron dense outer ring about 25 nm in diameter

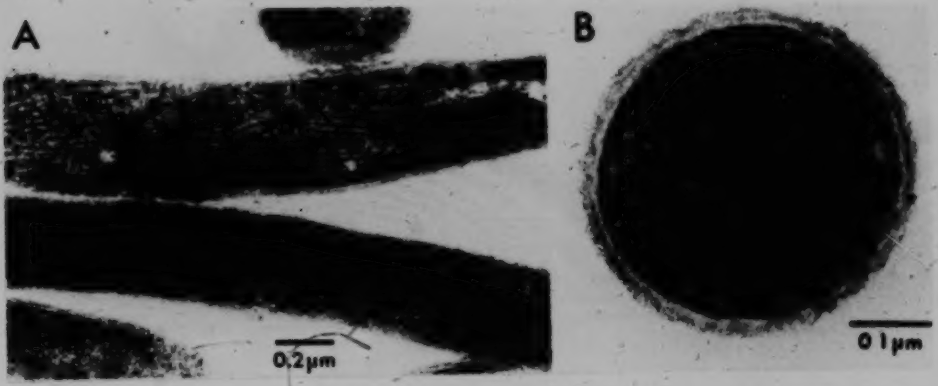

Figure V.1 Thin section electron micrographs. Thin section electron microscopy of longitudinal (A) and lateral (B) cross-sections of *E. coli* HB101/pFRD63 grown to stationary phase as described in Methods.

bounding a less dense core of 16 nm in diameter.

In situ lysis and negative staining electron micrscopy

The tubules were also observed in cells by negative staining. Spheroplasts prepared by lysozyme-EDTA treatment were lysed and washed on the microscope grid. This procedure removed much of the cytoplasmic material but the tubules remained bound to the cells (Figure V.2). Under these conditions the tubules extrude from breaks in the cell

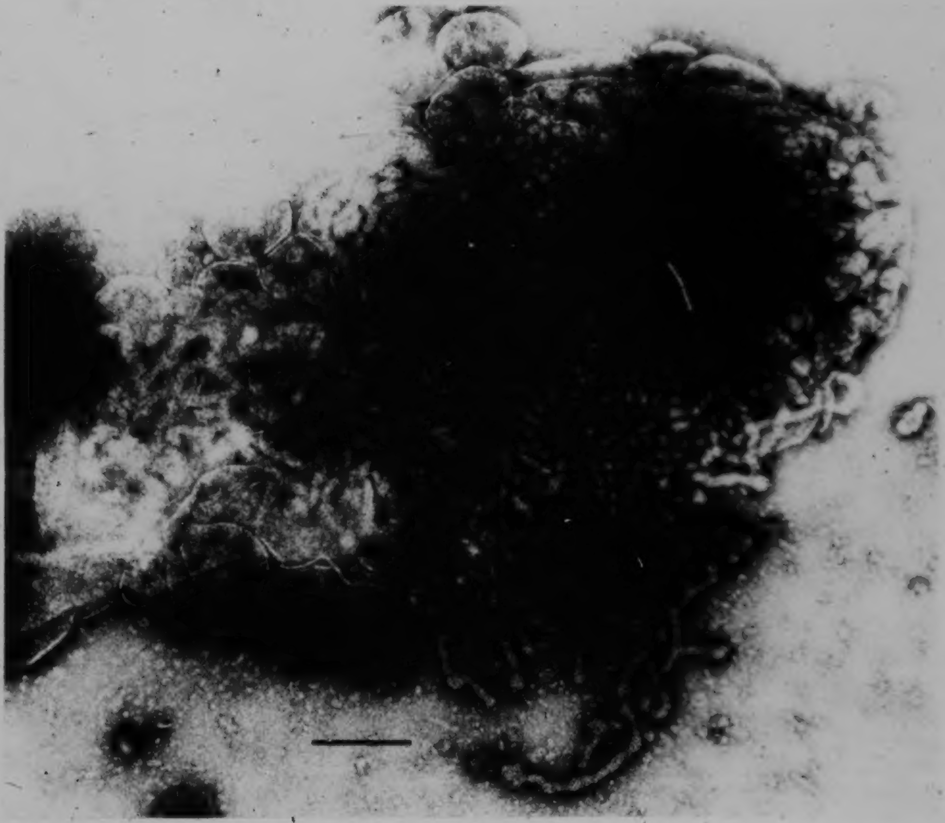

Figure V.2 Negative stain electron micrograph. Negative stain electron microscopic view of a cell after "*in situ*" lysis as described in Methods. The bar represents 0.2µm.

envelope. The tubules pictured in Figure V.2 are covered with an array of the 40Å knobs which we have previously shown are the catalytic heads of fumarate reductase (Lemire *et al.*, 1983). Inspection of tubules released from the cell (Figure V.3A and B) indicates that the tubules reach lengths many times the length of an *E. coli* cell, that they branch at irregular intervals and are presumably attached to the cytoplasmic membrane (Figure V.3B). The arrows in Figure V.3A highlight the position of 0.1 μm vesicle like structures which are regularly spaced along the tubules at 2 μm intervals. As this distance is about the length of an *E. coli* it seems likely that the vesicles may be remnants of tubule attachment points at the poles of the bacterial cell.

Time course of tubule appearance

We have found that we can observe the appearance of fumarate reductase in the supernatant fraction after 150,000 xg, 90 min centrifugation of envelopes prepared by French press lysis. This activity is not soluble but resides in small fragments of the tubules, as seen in electron micrographs, which do not sediment under the conditions used. These fragments appear as a result of the lysis manipulations and provide a simple means of following cytoplasmic fumarate reductase activity and hence tubule formation. In Figure V.4 we plot the specific activity of fumarate reductase in the envelope and supernatant fractions as

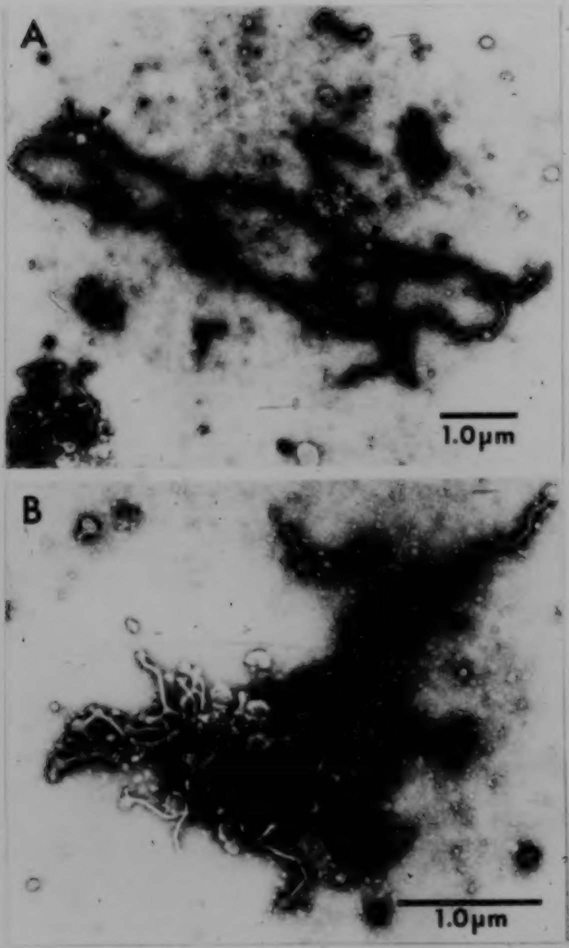

Figure V.3 Electron micrographs of tubules. Negative stain electron microscopic views of the tubules released by "*in situ*" lysis. The arrows in (A) indicate the position of a regularly spaced vesicular structure (see text) and (B) shows the tubules attached to membrane fragments.

a function of time after incubation under anaerobic growth conditions. Activity first appears in the membrane fraction, and only after a 6 to 8 hr lag, can activity be detected in the supernatant. This indicates that the tubules form only after the membrane approaches saturation with fumarate reductase. As seen in Figure V.4, saturation

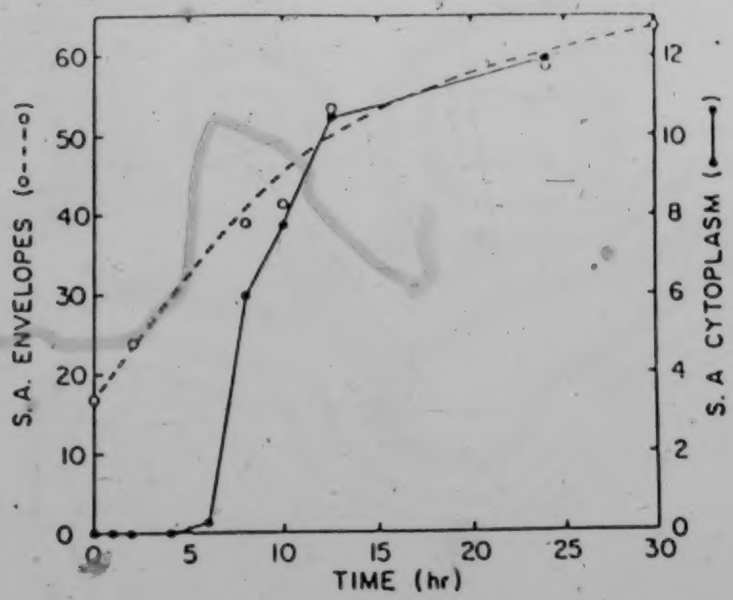

Figure V.4 Time course of fumarate reductase expression. Time course of appearance of fumarate reductase activity in the inner membrane fraction (o) and cytoplasmic fraction • of cells lysed by French pressure lysis followed by centrifugation at 150,000 xg for 90 min. Cells were grown overnight in rich medium (Lemire *et al.*, 1982), diluted 20-fold into glycerol-fumarate medium (Spencer and Guest, 1974) flushed with nitrogen, sealed and grown with slow stirring at 37°C. Samples were anaerobically removed at the indicated times, the cells harvested and washed once in 0.2 M potassium phosphate buffer pH 6.8 and lysed as described in (Lemire *et al.*, 1982).

of the membrane occurs when the specific activity of fumarate reductase approaches 65 units per mg protein. The level of reductase in the cell continues to increase with the excess enzyme localized in the tubules.

D. DISCUSSION

In this paper we have examined the effect of amplification of fumarate reductase on several aspects of *E. coli* membrane physiology. Experiments were carried out with stationary phase cells in which the enzyme was amplified 30-40 fold so that at least 50% of the membrane protein was fumarate reductase. Initial experiments were designed to see if amplification displaced other membrane-associated enzymes. We quantitated the levels of several membrane-bound enzymes and found that they were present in the control and fumarate reductase enriched membranes at about the same levels per weight of cells, although the specific activities fell, in the enriched membranes, due to dilution by large amounts of fumarate reductase. These results are in contrast to the work of Kung and Henning (Kung and Henning, 1972) who found that co-induction of several membrane-bound dehydrogenases resulted in substantial increases in the amount of soluble activity in the cytoplasm. They inferred from their results that the membrane could accommodate only very limited amounts of protein. The cell membrane may indeed have a limited capacity, but this is much greater than previously believed. It would appear that for at least fumarate reductase, the cell copes with the excess membrane protein by synthesizing a novel lipid protein complex rather than accumulating soluble enzyme in the cytoplasm.

Interestingly, although NADH dehydrogenase was reduced only about 30%, the NADH oxidase electron transport chain activity was completely absent in the amplified membranes. This could result either from a total loss of one of the chain components (cytochrome levels were normal, data not shown) or more likely from a defect in lipid-protein or protein-protein interactions caused by large amounts of reductase in the membranes. The glycerol-3-phosphate to fumarate chain is not disturbed by excess fumarate reductase and functions normally as judged by the normal doubling times of cells carrying a *frd* plasmid.

The cells respond to the excess membrane protein biosynthesis by a regulated increase of membrane phospholipid biosynthesis such that the lipid to protein ratio remains nearly constant at 0.4. The phospholipid composition of anaerobically grown *E. coli* HB101/pBR322 was similar to that reported for aerobic cells grown to stationary phase (Ames, 1968), except for the absence of cardiolipin and the presence of a phospholipid which migrated with R_f values consistent with acyl phosphatidylglycerol (Olsen and Ballou, 1971). The major change in phospholipid composition upon amplification of fumarate reductase was the disappearance of the acyl phosphatidylglycerol and the appearance of cardiolipin. In stationary phase the cardiolipin was 15% of the inner membrane phospholipid, considerably higher than the 5% normally seen with *E. coli* (Jain and Wagner, 1980). The increase in cardiolipin may be a specific response to fumarate reductase amplification and this is under investigation. The increase in cardiolipin biosynthesis is however reminiscent of the increase associated with bacteriophage M13 coat protein accumulation (Chamberlain and Webster, 1976). After infection of *E. coli* with bacteriophage M13, the virus assembles within the inner membrane of *E. coli* and the bacteria stores large quantities of the major coat protein (gene 8 protein) of the virus in the cytoplasmic membrane. We did not observe tubule formation in the M13-infected cells (data not shown).

The cells accommodated the extra membrane lipid and protein by synthesizing a tubular structure composed of a 16 nm diameter lipid-protein core surrounded by a 9 nm electron dense outer ring composed of an array of the catalytic subunits of fumarate reductase. The tubules are attached to the cytoplasmic membrane, appear to branch and grow to lengths of several microns, indicating that they must traverse the bacterial cell several times. The uniformly spaced vesicular structures seen on the tubules (Figure V.3A) may correspond to attachment sites at the poles of the bacterial cell. A tubular structure rather than a vesicular structure is probably the result of protein-protein interactions between adjacent reductase molecules and reconstitution studies are in progress to examine this possibility in vitro.

We do not know if tubule formation is unique to fumarate reductase or whether amplification of other membrane enzymes will induce similar structures. There are indications that amplification of some membrane enzymes may not occur (Mobley *et al.*, 1983) perhaps due to their inability to induce the necessary changes in phospholipid biosynthesis.

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VI. A NOVEL MUTANT OF *Escherichia coli* FUMARATE REDUCTASE DECOUPLED FROM ELECTRON TRANSPORT[1]

A. INTRODUCTION

Fumarate reductase is a complex, membrane-bound iron sulfur flavoenzyme that serves as the terminal electron transfer enzyme when *E. coli* is grown anaerobically with fumarate as the electron acceptor (Ingledew and Poole, 1984). The enzyme is composed of two distinct domains: a membrane extrinsic catalytic domain comprised of the *frd*A and B polypeptides of 69 and 27 kD, respectively, and a membrane intrinsic domain consisting of the *frd*C and D subunits of 15 and 13 kD, respectively (Weiner *et al.*, 1984c). Two forms of the enzyme can be isolated: a tetrameric holoenzyme composed of equimolar amounts of each subunit and a catalytic dimer composed of the *frd*A and B subunits (Dickie and Weiner, 1979; Lemire *et al.*, 1982). By analogy with the *E. coli* succinate dehydrogenase, the active site of fumarate reductase is located in *frd*A, the flavin-containing subunit (Wood *et al.*, 1984). The iron-sulfur centers are located in the *frd*B subunit (Cole *et al.*, 1982) and the *frd*C and D polypeptides do not contain any known redox centers. We have clearly shown that the *frd*C and D polypeptides not only anchor the catalytic subunits to the membrane surface (Lemire *et al.*, 1982), but also induce an optimal conformation in the catalytic dimer as reflected by increased stability and modulated turnover of the holoenzyme (Weiner *et al.*, 1984a). The enzyme is believed to accept reducing equivalents (electrons) from a reduced b-type cytochrome buried within the membrane. However, the pathways for electron within the electron transport chain and within the enzyme itself remain to be firmly established.

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[1] A version of this chapter has been accepted for publication. Weiner, J.H., R. Cammack, S.T. Cole, C. Condon, N. Honoré, B.D. Lemire and G. Shaw. 1986. *Proc. Natl. Acad. Sci. U.S.A.* (in press).

In contrast to all previously characterized mutants of the *frd* operon, which have been localized to the *frd*A gene (Lambden and Guest, 1976), we now report the isolation and analysis of a mutant within the *frd*C subunit. Mutant enzyme is unable to accept electrons from either the physiological donor or the quinone analog 2,3-dimethyl-1,4-naphthoquinone (DMN) and yet functions normally with the artifical electron donor reduced benzyl viologen (BVH$^{\cdot}$). The differential utilization of electron donors indicates the presence of two spatially distinct sites at which reduction can occur.

B. MATERIALS AND METHODS

Materials

DMN was the gift of Dr. A. Kröger, Phillips University, Marburg FRG.

Strains and plasmids

These are described in Table VI.1.

Table VI.1

Strains and plasmids

Bacteria	Genotype	Source
E. coli HB101	F^-, *hsd*R, *hsd*M, *pro*, *leu*, *lac*, *gal*, *thi*, *rec*A, *rps*L	G.McFadden (U. of Alta)
E. coli GS1	F^-, *hsd*R, *hsd*M, *pro*, *leu*, *lac*, *gal*, *thi*, *rec*A, *rps*L, *frd*A4	laboratory collection
E. coli GS19	F^-, *hsd*R, *hsd*M, *pro*, *leu*, *lac*, *gal*, *thi*, *rec*A, *rps*L, *frd*C1	this study

Plasmids	Genotype	Vector	Source
pFRD84	Ap, *frd*$A^+B^+C^+D^+$	pBR322	(a)
pFRD117	Ap, *frd*A^+B^+	pBR322	(a)
pFRD215	Ap, *frd*C^+D^+	pBR322	(b)
pFRD704	Ap, *frd*C^+	pBR322	(c)
pFRD38	Ap, *frd*D^+	pUC8	G. Cecchini (UCSF)
pFRD300	Ap, *frd*$A^+B^+C^-D^+$	pUC13	~~this s~~tudy

(a) (Lemire *et al.*, 1982); (b) pFRD215 was generated by restriction endonuclease SacII digestion of pFRD84 followed by ligation with T4 DNA ligase (Lemire *et al.*, 1982); (c) pFRD704 is a Bal31 exonuclease generated deletion of pFRD215 lacking all but the first 36 nucleotides of *frd*D; Ap=Ampicillin resistant.

Growth of cells and preparation of inverted membrane vesicles

All cells were grown at 37°C. For anaerobic growth, glucose-peptone, glycerol-fumarate (GF) or glycerol-nitrate media were prepared as described previously (Weiner *et al.*, 1984b). For growth with H_2(g) (H_2/fum medium), the glycerol-fumarate medium was supplemented with 0.5% peptone and sparged with H_2-CO_2 (95:5). Flasks were filled to the top with medium and stoppered. Cells were inoculated at 1%

(v/v).from an aerobic overnight culture on L-broth (Miller, 1972). Amino acids, vitamins and ampicillin (100 µg/ml) were added as needed. Cultures were harvested after 24 h. Membrane vesicles were prepared by French pressure lysis (Aminco Inst. Co. Rockville, Md.) as described previously (Lemire *et al.*, 1982).

Isolation of mutant GS19

A mid-log phase culture of *E. coli* HB101 grown on L-broth was mutagenized with 40 µg/ml of N-methyl-N^1-nitro-N-nitrosoguanidine for 7.5 min. at 37°C in 0.1M citrate buffer, pH 5.5 (Miller, 1972). The cells were washed with 0.1M KPi buffer, pH 7.0, resuspended in L-broth, grown overnight and then transferred to GF medium for 36 h of aerobic growth to eliminate auxotrophs. The culture was exposed to 1 cycle of ampicillin enrichment (100 µg/ml).in anaerobic GF medium, and subsequently plated on L-broth plates from which single colonies were replicated to duplicate GF plates incubated either anaerobically or aerobically. Each mutant unable to grow anaerobically was transformed with plasmid pFRD84 (Lemire *et al.*, 1982) and ampicillin resistant transformants were selected. Mutants complemented by pFRD84 were *frd* mutants. Cell free extracts from the *frd* mutants were assayed for fumarate dependent benzyl viologen oxidase activity.

Cloning of a mutant *frd* operon

Chromosomal DNA was digested with endonuclease HindIII and fragments of about 4.9 kB, purified by gel electrophoresis and NACS column chromatography (BRL Bethesda Md.). Purified DNA was ligated into dephosphorylated HindIII cut pUC13 vector DNA (Pharmacia P-L, Dorval, P.Q.) and transformed into competent GS1 (*frd*A$^-$) cells. Ampicillin resistant transformants were selected and tested for growth on GF plates incubated anaerobically. A few poorly growing colonies were detected and their plasmids miniscreened (Birnboim and Doly, 1979). pFRD300 contained the mutant fumarate reductase operon on the expected HindIII fragment (Lohmeier *et al*., 1981).

DNA sequence analysis

This was performed as described previously except that in some reaction mixtures GTP was replaced by ITP (Cole, 1982).

Fumarate reductase assays

The reduced benzyl viologen (BVH$^{\cdot}$) assay was as described (Dickie and Weiner, 1979; Robinson and Weiner, 1982). The reduced DMN (DMNH$_2$) assay was a modification of the method of Kröger and Innerhofer (Kröger and Innerhofer, 1976). Assays were performed in a final volume of 1.1 ml of N$_2$-saturated 50 mM KPi, pH 8.0. The assay contained 0.5 mM DMN and 1 mM fumarate. Reduction of

DMN was accomplished by the addition of 2mM $NaBH_4$. The reaction was started by the addition of 5-20 units of fumarate reductase (BVH˙ oxidase activity) and the increase in absorption at 270 nm followed. 1 unit corresponds to 1 µmole of $DMNH_2$ oxidized per min at 24°C. The extinction coefficent of DMN at 270 nm is 16,000 M^{-1} (Kröger and Innerhofer, 1976).

Quinacrine fluorescence quenching

Assays were performed as described by Jones (Jones, 1979) using either H_2 or N_2 saturated buffers. The reaction was initiated with 0.5 µmoles of fumarate. 2-n-heptyl-4-hydroxyquinoline N-oxide (HOQNO) was used at 6 µM.

Protein determination

This was estimated by the modified Lowry procedure (Markwell *et al.*, 1978).

Limited proteolysis analysis

V8 protease digestion was performed on gel slices as described by Cleveland (Cleveland *et al.*, 1977). Digests were run on 15% SDS-polyacrylamide slab gels with low molecular weight protein standards (BRL Ltd.). Gels were scanned on a Joyce Loebl chromoscan 3 densitometer. V8 protease was the gift of Dr. L.B. Smillie, University of Alberta, Edmonton, Canada.

EPR spectroscopy

EPR spectra were recorded on a Varian E4 spectrometer fitted with a E1020 microwave bridge and Oxford Inst. ESR9 liquid helium flow cryostat. Samples were analyzed in cylindrical quartz cuvettes of 3.5 mm internal diameter.

C. RESULTS

Characterization of GS19

Mutagenesis of HB101 was performed to isolate fumarate reductase mutants. Mutant GS19 was unique in that although unable to grow anaerobically on glycerol with fumarate as electron acceptor, it had normal fumarate-dependent BVH˙ oxidase activity. The mutant grew anaerobically on glycerol-nitrate, glucose-peptone and, surprisingly, on H_2-fum media indicating that it was not defective in an essential electron transport component (Table VI.2). Measurements of the menaquinone, ubiquinone and b-type cytochrome levels confirmed that these components were present in normal amounts (data not shown).

Complementation studies

Plasmids carrying all or part of the wild-type *frd* operon were transformed into GS19 and anaerobic growth on GF medium assayed. pFRD84 carrying a complete *frd* operon could complement the mutation (Table VI.2). Surprisingly, both pFRD117 which expresses the *frd*A and B polypeptides and

Table VI.2

Growth studies on anaerobic medium

Medium	Strain	Klett Units
Glycerol-fumarate	HB101	64
	GS19	3
	GS19/pFRD84	109
	GS19/pFRD117	100
	GS19/pFRD215	95
	GS19/pFRD704	105
	GS19/pFRD38	10
	HB101/pFRD84	88
	HB101/pFRD300	15
Glycerol-nitrate	HB101	118
	GS19	91
	HB101/pFRD84	88
	HB101/pFRD300	142
Hydrogen-fumarate	HB101	202
	GS19	165

All growth experiments were performed at 37°C. Cultures were inoculated with a 1% (v/v) inoculum of an overnight aerobic L-broth culture into 125 ml Klett flasks filled to the top and stirred slowly on a magnetic stirrer. When hydrogen was used the flasks were continuously sparged with filtered gas through a serum cap. Readings were taken after 24 hr. of growth with a Klett colorimeter using a red filter. Expression of *frd*D on plasmid pFRD38 was under tryptophan control and was induced with 10 μg/ml 3-indolylacetic acid.

pFRD215 which produces the *frd*C and D subunits could complement (Table VI.2). Furthermore, pFRD704 which carries *frd*C alone could complement whereas pFRD38 bearing *frd*D could not.

Limited proteolysis

Fumarate reductase polypeptides from the mutant (pFRD300) and wild-type (pFRD84) enzyme were compared by limited proteolysis with V8 protease. The peptide profiles obtained for each of of the two *frd*C-D patterns (the anchor polypeptides were not resolved in this system) show several differences, of which the most striking is a novel peptide in the mutant sample (Figure VI.1). The fumarate reductase proteins are relatively resistant to digestion by V8, and the protease was employed at relatively high concentration (see Figure VI.1). Under these conditions V8 protease can cleave

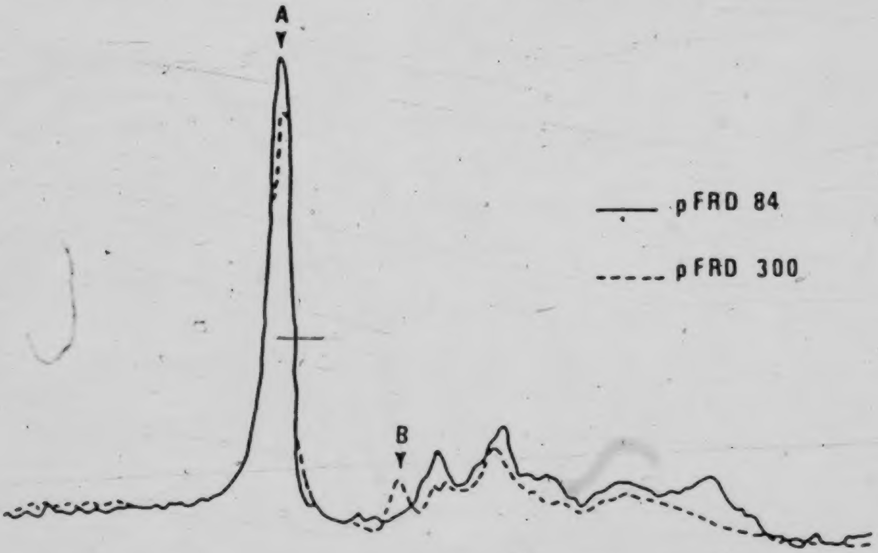

Figure VI.1 Limited proteolysis of *frd*C-D. Limited proteolysis of wild-type and mutant *frd*C-D. Excised *frd*C-D polypeptide band from 60 μg purified tetramer was treated with 0.5 μg V8 protease and analyzed by SDS-polyacrylamide gel electrophoresis. Densitometric scans of lanes from a 15% gel are shown. Solid line, pFRD84; dashed line, pFRD300. A) undigested C-D protein at 15 kD. B) Novel peptide in mutant C-D profile at 10kD.

after histidine in addition to normal cleavage after glutamate (Lau *et al.*, 1985).

Cloning and Sequence analysis

To determine the exact nature of the mutation in GS19, the mutant *frd* operon was cloned into pUC13, generating plasmid pFRD300. The genetic properties of pFRD300 resembled those of GS19 except that in a partial diploid a negative dominance phenotype was observed when grown on glycerol-fumarate medium (Table VI.2). To resolve the discrepancy in the complementation experiments and to localize the mutation the DNA sequences of *frd*A, B and C were determined and compared to those of the wild-type (Cole *et al.*, 1982; Cole, 1982; Grundström and Jaurin, 1982). A single base difference was observed, namely an A to G transition at position 3575. This changes His82 in the second transmembranal alpha-helix of the *frd*C polypeptide to arginine.

Catalytic properties of the mutant enzyme

As shown in Table VI.3 the BVH$^+$ oxidase activity of fumarate reductase in lysates of GS19 is comparable to that of the parental strain. Furthermore, the enzyme levels of cells harboring either the cloned wild-type or mutant operons are similar. Using the BVH$^+$ assay, the Km for fumarate was the same for both enzymes (425 μM). However, the specific activity of purified mutant holoenzyme was 3.5 fold higher

Table VI.3

Fumarate reductase activity of membranes and extracts

Enzyme		BV Activity		DMN Activity	
Strain	Source	u/mg -HOQNO	u/mg +HOQNO	u/mg -HOQNO	u/mg +HOQNO
HB101	memb. bound	3.6	3.5	ND	ND
GS19	memb. bound	3.7	3.6	ND	ND
HB101/pFRD84	memb. bound	28.3	28.9	0.424	0.026
HB101/pFRD300	memb. bound	48.6	44.7	0.024	0.008
HB101	det. extract	4.9	4.9	0.042	0.008
GS19	det. extract	8.7	8.8	ND	ND
HB101/pFRD84	det. extract	37.9	39.6	0.502	0.029
HB101/pFRD300	det. extract	93.0	91.2	0.030	0.006

Activities (u/mg) are reported as µmoles of reduced benzyl viologen or reduced DMN oxidized per min. per mg. protein. Membrane bound activity was measured using French press vesicles. Detergent extracted enzyme was prepared by 1% octaethyleneglycol mono n-dodecyl ether (($C_{12}E_8$) Nikkol Chemicals, Tokyo, Japan.) extraction exactly as reported for Triton X-100 (Lemire *et al.*, 1982). HOQNO was used at 6 µM. ND=not determined.

than wild-type holoenzyme (550 µ/mg vs. 150 µ/mg) and approached the high specific activity of catalytic dimer (Cole, 1982). Kröger and colleagues have shown that the quinone analogue $DMNH_2$ can serve as a donor of reducing equivalents to fumarate reductase of *Wollinella succinogenes* in the presence of the b-type cytochrome subunit (Kröger and Innerhoffer, 1976). We have utilized $DMNH_2$ oxidase activity to characterize fumarate reductase of *E. coli* and find that this activity is retained upon solubilization and purification of holoenzyme but is completely absent when the *frd*C and D subunits are removed during purification of the catalytic dimer. As shown in Table VI.3 the $DMNH_2$ oxidase activity of membrane-bound or purified wild-type holoenzyme is inhibited by HOQNO whereas the BVH˙ oxidase activity is resistant. The mutant enzyme possesses a greatly diminished $DMNH_2$ oxidase activity. Thus with respect to turnover and $DMNH_2$ activity the mutant holoenzyme resembles isolated catalytic dimer.

Physical properties of mutant enzyme

Chromosomal or plasmid-coded mutant fumarate reductase is membrane-bound and not released by salt or EDTA washes. This indicates that the mutation in *frd*C, one of the anchor subunits, does not alter membrane association of the catalytic dimer. The subunit molecular weights and mole ratios are identical to the wild-type. The isoelectric points and the V8 protease digestion patterns of the *frd*A and B properties of the mutant holoenzyme paralleled those of the wild-type holoenzyme (Weiner *et al.*, 1984c).

Electron transfer properties

The greatly diminished ability of the mutant enzyme to oxidize $DMNH_2$, suggested that the mutation decoupled fumarate reductase from the electron transport chain. This decoupling is manifested as an impaired fumarate-dependent protonmotive force formation, as measured by quinacrine fluorescence quenching. Inverted mutant membrane vesicles, incubated with glycerol-3-phosphate do not exhibit fumarate-dependent quinacrine quenching (data not shown). Membrane vesicles from HB101 substantially quench quinacrine fluorescence in a H_2 and fumarate-dependent, HOQNO-sensitive manner (Figure VI.2). In contrast, GS19 membranes exhibit much

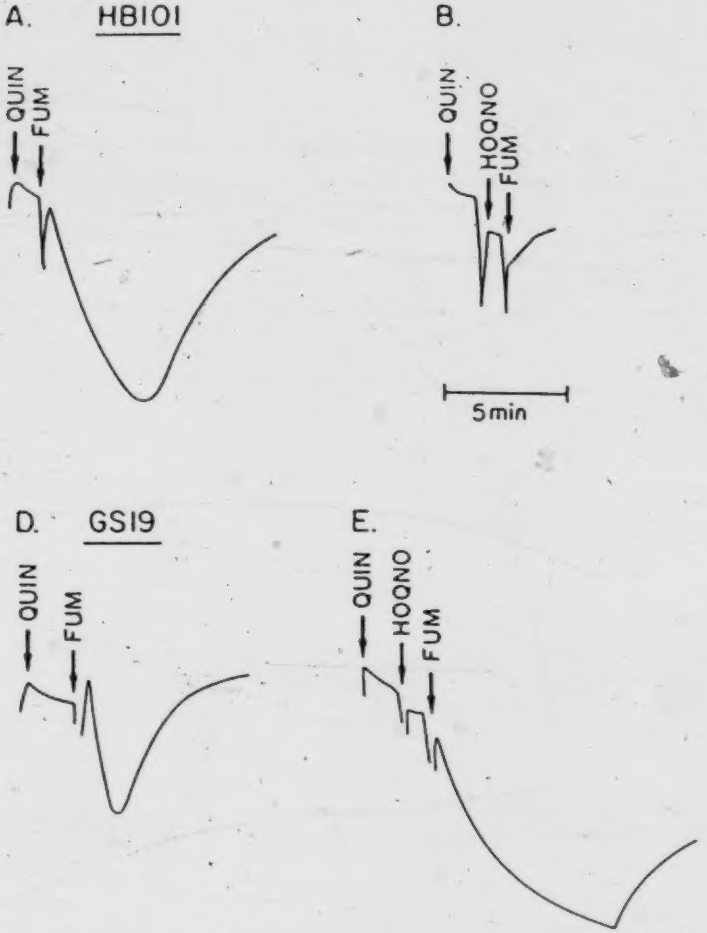

Figure VI.2 Fluorescence quenching. Quinacrine fluorescence quenching. A) French press vesicles prepared from *E. coli* HB101 (1.62 mg. protein in 2.5 ml of assay buffer) were saturated with H_2 (g) for 18 min prior to assay. Quinacrine (10 μmoles) and fumarate (0.5 μmoles) were added at the indicated times. B) as in A) except 6 μM HOQNO was added. C) as in A) except vesicles were prepared from *E. coli* GS19. D) as in B) except vesicles prepared from GS19.

less quenching and this quenching is HOQNO-resistant. In fact, the presence of HOQNO amplifies the quenching suggesting the inhibitor blocks proton leakage pathways in the mutant membranes. We attribute the quenching observed with mutant membranes to the vectorial mechanism of hydrogenase and not to the vectorial action of the electron transport chain (Jones, 1980). That fumarate-dependent oxidation of NADH or dihydroorotate (Newton *et al.*, 1971) is totally absent in the mutant membranes supports this.

EPR properties

French press membrane vesicles and fumarate reductase tetramer from both mutant and wild-type cells were examined by EPR spectroscopy. The mutant enzyme exhibited all the features previously described for the iron-sulfur centers of wild-type fumarate reductase (Cammack *et al.*, 1985a; Simpkin and Ingledew, 1985). In the oxidized state (Figure VI.1A), it displayed a signal at $g = 2.02$ due to center 3, which is probably a [3Fe-xS] cluster. On reduction with succinate or brief treatment (1 min) with 2 mM dithionite, an axial spectrum at $g = 2.03, 1.93$ was observed, due to the [2Fe-2S] center 1. On further incubation with 2 mM dithionite an additional reduction of the enzyme took place which was observed as an increase in the spin-lattice relaxation rate of the $g = 1.93$ signal. This reduction is attributed to center 2, which, from the amino acid sequence, is likely to be a [4Fe-4S] cluster. In our experiments, the reduction of

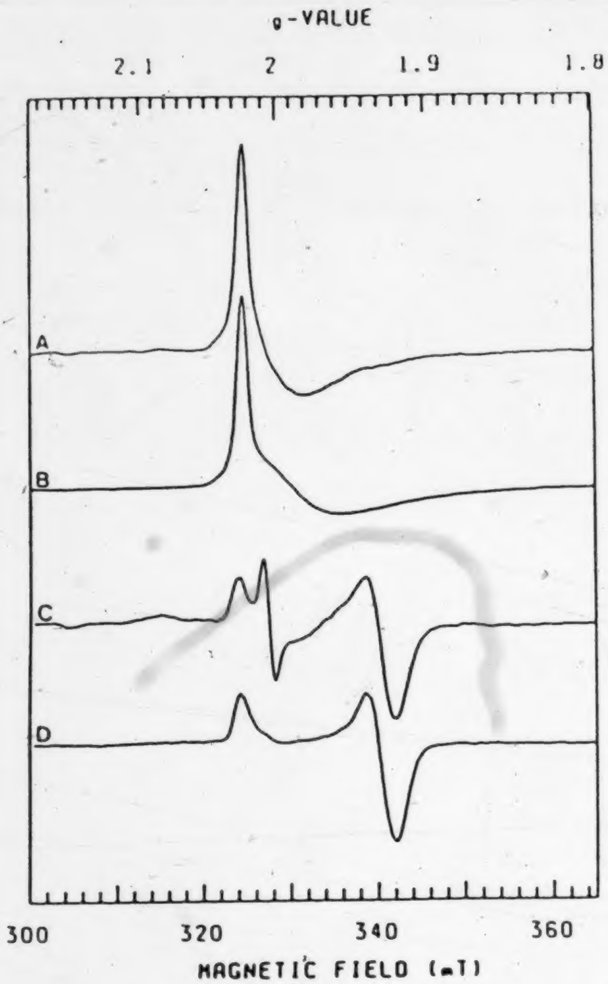

Figure VI.3 EPR spectra of fumarate reductase. EPR spectra of fumarate reductase in French press membrane vesicles (A) and (C) *E. coli* mutant HB101/pFRD300 and (B) and (D) wild-type HB101/pFRD84. (A) and (B) were in the oxidized state and show the spectra of center 3 at g = 2.02. (C) and (D) were reduced with 5 mM dithionite at 20°C for 2 min and show the spectra of center 1 at g = 2.03, 1.93. Spectrum (C) also shows the spectrum of a flavin radical at g = 2.004. Spectra were recorded at 11K; microwave power was 2 mW for (A) and (B), 2 μW for (C) and (D).

center 2 does not contribute significantly to the intensity of the g = 2.03, 1.93 signal, but corresponds to the appearance of an extremely broad signal centered around g =

1.95 (Cammack *et al*., 1985a; Cammack *et al*., 1985b). A broad signal, of somewhat different lineshape, was also observed during reduction of center 2 in membranes of the mutant.

Although the EPR spectra observed with the mutant enzyme were qualitatively similar to those observed in the wild-type enzyme, there were differences observed during the time-course of reduction by dithionite. Firstly, a flavin radical signal (Figure VI.3C) was more intense in the mutant enzyme, and remained for several minutes before it disappeared by further reduction. Secondly, the reduction of center 1 was unusually slow, requiring 10 minutes for completion. This suggests that the normal pathway for reduction of the fumarate reductase by dithionite is absent in the mutant.

D. DISCUSSION

Fumarate reductase, a complex iron sulfur flavoenzyme, contains at least three prosthetic groups capable of undergoing oxidation-reduction reactions (Ingledew and Poole, 1984; Weiner *et al*., 1984c). These include two or three iron sulfur centers, likely located in the *frd* B subunit (Cole *et al*., 1982), and a covalent flavin cofactor in the *frd*A subunit (Weiner and Dickie, 1979). Here, we report the isolation and characterization of an unusual mutation that decouples fumarate reductase from the electron transport chain and prevents anaerobic growth of *E. coli* on glycerol-fumarate medium. The mutation is a single A to G transition in *frdC* changing His82, within a proposed transmembranal alpha-helix, to arginine. In our model for the topography of the anchor subunits (Weiner *et al*., 1984c), this His residue is located close to the cytoplasmic surface of the membrane and could be expected to interact with the catalytic subunits. Although the catalytic subunits of *E. coli* and *Proteus vulgaris* fumarate reductase are very highly conserved, the anchor subunits diverge considerably in sequence, particularly in the hydrophobic transmembranal segments. Interestingly, a three residue sequence containing His82 is conserved, substantiating the importance of this residue (Honoré and Cole, unpublished).

The properties of the mutant fumarate reductase suggest the presence of two distinct sites at which reduction of the enzyme can occur (Figure VI.4). The electron transport chain normally carries electrons from hydrogenase or the primary dehydrogenases, to fumarate reductase in a cytochrome-dependent manner. We suggest that the physiological donor and $DMNH_2$ use the same site to reduce the enzyme. This site is sensitive to HOQNO and requires the presence of the *frd*C and D subunits. Reducing equivalents are subsequently translocated within the enzyme to the iron sulfur centers and the flavin. The second pathway for reducing equivalents is represented by the $BVH^{\cdot}$ reaction site. This site can receive electrons from cytochrome-independent sources, is HOQNO resistant and does not require the

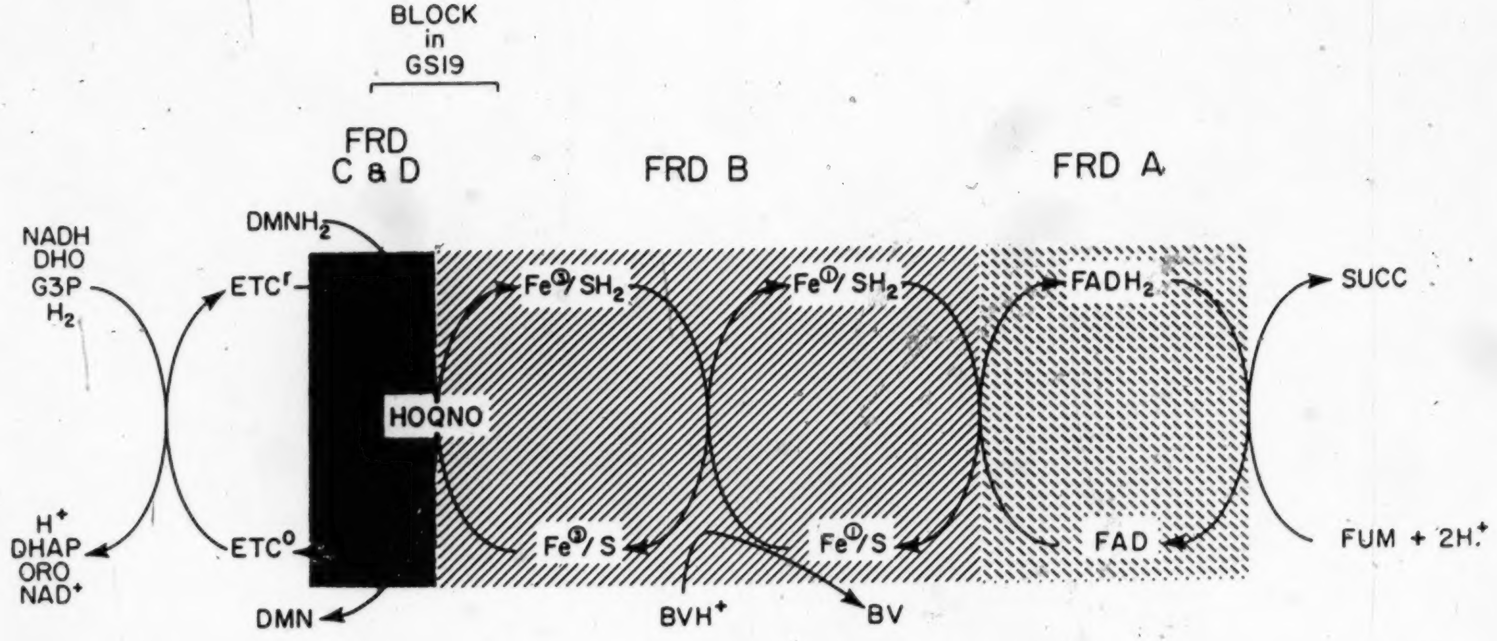
BLOCK in GS19
FRD C & D
FRD B
FRD A
NADH
DHO
G3P
H_2
H^+
DHAP
ORO
NAD^+
ETC^r
ETC^o
$DMNH_2$
DMN
HOQNO
$Fe^{③}/SH_2$
$Fe^{③}/S$
$Fe^{①}/SH_2$
$Fe^{①}/S$
BVH^+
BV
$FADH_2$
FAD
SUCC
$FUM + 2H^+$

Figure VI.4 A model for electron transport within fumarate reductase. Several primary dehydrogenases specific for NADH, dihydroorotate (DHO), glycerol-3-phosphate and hydrogen couple to fumarate reductase via the electron transport chain. ETC_o and ETC_r refer to the oxidized and reduced components, respectively. $DMNH_2$ apparently also couples to this site. At least 2 iron sulfur centers transfer electrons within *frd*B. In this model, center 3 precedes center 1 and is based on electrochemical potentials. (Cammack *et al.*, 1985a). The exact site of the benzyl viologen reaction is unknown but it must precede at least one iron-sulfur center because the reaction is inhibited by p-chloromercuribenzene sulfonate (Lemire and Weiner, unpublished). The apparent site of the block in GS19 is marked. ORO=orotate and DHAP=dihydroxyacetonephosphate.

*frd*C and D polypeptides. This pathway explains the cytochrome-independent electron transport from glycerol-3-phosphate to fumarate observed by Singh and Bragg in a heme deficent mutant (Singh and Bragg, 1976), the HOQNO-resistant protonmotive force observed by fluorescence quenching with H_2(g) in GS19 membranes, the ability of this mutant to grow with H_2 and fumarate and may also explain the complementation of GS19 by pFRD117. The mutant enzyme is unable to efficiently oxidize $DMNH_2$, although it uses BVH˙ normally. This suggests that the block precedes the site of BVH˙ oxidation.

Despite the absence of known redox groups in the *frd*C and D subunits, these polypeptides are essential for coupling the enzyme to the electron transport chain, or for receiving electrons from $DMNH_2$. Cecchini *et al.* (Cecchini *et al.*, 1984), have observed that fumarate reductase, functioning as a succinate dehydrogenase, will reduce the quinone analog 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone only when the *frd*C and D subunits are present, substantiating the role of the anchor polypeptides in electron flow. These data support our conclusion that the *frd*C and D polypeptides are intimately involved in the redox reactions of fumarate reductase, in addition to their physical role as anchor subunits.

Two unexpected aspects of this work should be noted. Firstly, although the mutation is in the *frd*C gene, a multicopy plasmid carrying *frd*AB could complement the mutation. We suggest a large excess of soluble catalytic dimer from the plasmid pFRD117 can phenotypically overcome the mutation by providing an accessible electron sink.

Secondly, cells harboring pFRD300 are unable to grow anaerobically on glycerol-fumarate medium, even though the chromosomal operon is normal. The negative-dominance effect is related to plasmid copy number (Lemire and Weiner, unpublished). Apparently excess aberrant enzyme is capable of interfering with the normal flow of electrons from the dehydrogenase to the wild-type chromosomally-coded fumarate reductase. Both of these results serve to highlight the dangers of doing genetic experiments with multicopy plasmids.

The mutation within the *frd*C polypeptide is reflected in changes to the redox properties of the iron sulfur and flavin cofactors located in the catalytic domain. The reduction of iron sulfur center 1 is slowed and the enzyme displays an unusually stable flavin semiquinone. Thus, the mutation is phenotypically transmitted from the membrane intrinsic anchor subunits to the extrinisic catalytic subunits. The high specific activity of the mutant holoenzyme with BVH^{+} as electron donor resembles that of the isolated catalytic dimer and suggests that the anchors regulate the turnover of the enzyme. We cannot yet distinguish if the anchors actively participate in electron transport or if they induce a conformation within catalytic dimer necessary for physiological electron transport. The transmission of conformational changes from intrinsic to extrinsic subunits may operate in other membrane-bound enzymes and may explain the phenotype of several F_0/F_1 ATPase mutants (Futai and Kanazawa, 1983).

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VII. CONCLUSIONS AND DISCUSSION

A. COMPARISON OF FUMARATE REDUCTASES AND SUCCINATE DEHYDROGENASES[1]

The fumarate-succinate oxidoreductases from various sources appear to be a highly conserved group of enzymes. The similarities between fumarate reductase from *E. coli* and mammalian succinate dehydrogenase are quite remarkable. Already, our understanding of fumarate reductase has contributed to the ongoing characterization of bovine succinate dehydrogenase (Singer and Johnson, 1985). The stability and ease of preparation of fumarate reductase have allowed rapid progress, but it is the genetics which will likely allow a more detailed characterization of the reductase. The vital role of succinate dehydrogenase in mammalian respiration encourages me to elaborate on the similarities of the enzymes in this family.

There appear to be two classes of oxidoreductases based on subunit composition. The first class consists of four subunit oxidoreductases while the other class is composed of three subunit enzymes.

FOUR SUBUNIT OXIDOREDUCTASES

Tables VII.1 and VII.2 list a number of properties associated with fumarate-succinate oxidoreductases. The four subunit class of enzyme includes the *E. coli* fumarate reductase, the *E. coli* succinate dehydrogenase, the beef heart mitochondrial succinate dehydrogenase and the *M. luteus* succinate dehydrogenase. Each of these has a 64-72,000 dalton subunit to which is covalently attached an FAD molecule as prosthetic group (Weiner and Dickie, 1979; Condon and Owen, 1982a; Singer *et al.*, 1955; Crowe and Owen, 1983). This subunit is also believed to contain the catalytic site.

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[1] Portions of this chapter have been published. Cole S.T., C. Condon, B.D. Lemire and J.H. Weiner. 1985. *Biochim. Biophys. Acta 811*:381-403.

The catalytic subunits of the *E. coli* fumarate-succinate oxidoreductases presumably derive from a common ancestral gene (Weiner *et al.*, 1986). Fumarate reductase and succinate dehydrogenase show marked homology at the amino acid level but are immunologically distinct (Wood *et al.*, 1984). This is also true of fumarate reductases and succinate dehydrogenases of other sources (Unden and Cole, 1983; Condon and Owen, 1982b). For example, succinate dehydrogenases from sources other than the members of the *Micrococcus* genus did not crossreact with antibody specific to *M. luteus* succinate dehydrogenase (Crowe and Owen, 1983). These enzymes reversibly catalyze the same reaction, differ markedly in their affinities for fumarate and succinate and are subject to different genetic regulation (Robinson and Weiner, 1982; Cole *et al.*, 1985).

A 27-30,000 dalton subunit, necessary for catalytic activity, is believed to be the location of the iron sulfur clusters. The enzymes may differ in the organization of the

Table VII.1

Midpoint Potential of Succinate Dehydrogenases and Fumarate Reductases from Various Sources

		Centre			Ref.
		1	2	3	
Fumarate reductase	*E. coli*	-20	-330	-70	a
	E. coli	-50	-285	-30	b
	W. succinogenes	-59	--	-24	c
Succinate dehydrodenase	*E. coli*	+10	-175	+65	d
	M. luteus	+70	-295	+10	e
	bovine mito.	0	-26	+(60-120)	f

Potentials are in millivolts; mito=mitochondria; a) (Cammack *et al*., 1985) b) (Simpkin and Ingledew, personal communication) c) (Unden *et al*., 1984) d) (Condon *et al*., 1985) e) (Crowe *et al*., 1983) f) (Ohnishi and Salerno, 1982).

iron-sulfur centres. The subunit location of the redox clusters is uncertain, the B-polypeptides are strongly implicated by analogy to other systems. The *frd*B subunit of fumarate reductase has three arrangements of cysteine residues homologous with known cluster binding sites. Analogous sequences are found in *sdh*B of *E. coli*, but for the binuclear site (centre 1), the third cysteine (residue 65 of *frd*B) is replaced by aspartate (Darlison and Guest, 1984). If asp62 of *sdh*B were a ligand it would probably cause the potential of the centre to become more positive (Johnson and Holm, 1978). This proposal is certainly in agreement with the experimental observations on the relative potentials of the centres (see Tables VII.1 and VII.2). For both enzymes, a region homologous with the four iron ferredoxin binding sequences (residues 203 to 215 for *frd*B and 204 to 216 for *sdh*B) also lacks one of the

Table VII.2

Comparison of fumarate reductases and succinate dehydrogenases

Enzyme	Source	Subunits Number	Subunits [1]Mol.Wt.(kDa)	Associated Cytochrome	Covalent Flavin	References
Fumarate Reductase	*E. coli*	4	66,27,15,13[2]	--	+	1,2,3,4
	W. succinogenes	3	79,30,25[3]	b_{560} (-20mV)	+	5,6,7
Succinate dehydrogenase	*E. coli*	4	64,27,14,13[2]	b-type	+	8,9
	B. subtilis	3	65,28,19	b_{558}	+	9,10,11,12,13
	M. luteus	4	72,30,17,15	b-type	+	14
	[1]B.H. mitochondria	4	70,27,13.5,7.0	b_{560}	+	15,16,17,18

[1] abbreviations: B.H.=beef heart; Mol.Wt.(kDa)=molecular weight in kilodaltons.
[2] molecular weights calculated from the sequences.
[3] ratio of the three subunits is 1:1:2.
1) Cole, 1982. 2)Grundström and Jaurin, 1982. 3) Cole *et al.*, 1983. 4) Weiner and Dickie, 1979. 5) Albracht *et al.*, 1981. 6) Kenney and Kröger, 1977. 7) Unden *et al.*, 1980. 8) Condon *et al.*, 1985. 9) Condon and Owen, 1982a. 10) Hederstedt and Rutberg, 1981. 11) Hederstedt, 1980. 12) Hederstedt *et al.*, 1979. 13) Hederstedt *et al.*, 1985. 14) Crowe and Owen, 1983. 15) Capaldi *et al.*, 1977. 16) Hatefi and Galante, 1980. 17) Hemmerich *et al.*, 1969. 18) Kearney and Bernath, 1960.

constituent cysteines (Darlison and Guest, 1984; Cole *et al.*, 1982). Further elucidation of molecular organization of the iron sulfur clusters may well shed light on the differences between the two *E. coli* enzymes.

All of the four subunit enzyme complexes are believed to contain three iron sulfur clusters; a [2Fe-2S] cluster, a [3Fe-xS] cluster and a [4Fe-4S] cluster referred to as centres 1, 3 and 2 respectively (Singer and Johnson, 1985; Johnson *et al.*, 1985a; Johnson *et al.*, 1985b; Morningstar *et al.*, 1985; Crowe *et al.*, 1983; Cammack *et al.*, 1985). The [3Fe-xS] centre has only been shown in the beef heart succinate dehydrogenase and in the *E. coli* fumarate reductase but it is believed to exist in the other forms by analogy. The measured potentials of these centres vary considerably as seen in Table VII.1, but the similarities between complexes suggest that each of the iron sulfur clusters might perform analogous functions in the different enzymes.

Two small hydrophobic subunits of between 7,000 and 17,000 daltons are apparently necessary for membrane association (Lemire *et al.*, 1982; Condon *et al.*, 1985; Ackrell *et al.*, 1980; Crowe and Owen, 1983). Interestingly, the C and D polypeptides of the *E. coli* enzymes are of nearly identical sizes. The sequences of these subunits are not conserved. Rather, structural similarities exist in the distribution of hydrophilic and hydrophobic domains, such that the hypothesized organization of the dimer across the membrane is similar in each case (Grundström and Jaurin, 1982; Weiner *et al.*, 1984a; Wood *et al.*, 1984). Since *frd*C and *frd*D modulate the catalytic activity of the enzyme, it is tempting to speculate that the functional specificities of the two flavoenzymes are controlled by the anchors. These subunits may function as cytochromes, in quinone binding and in membrane association (Hatefi and Galante, 1980; Yu and Yu, 1980).

Of these four tetrameric enzyme complexes, three have been reported to be associated with a b-type cytochrome (see Table VII.2). An extensive search for a cytochrome associated with the *E. coli* fumarate reductase has been unsuccessful (Cole *et al.*, 1985). Although a b-type cytochrome is present in the membrane and participates in the fumarate electron transport chain (Singh and Bragg, 1976), it does not seem to be part of the holoenzyme. The *E. coli* succinate dehydrogenase was shown to contain a b-type cytochrome, possibly cytochrome b_{556} (Condon *et al.*, 1985; Murakami *et al.*, 1984). The succinate dehydrogenase from *M. luteus* also contained a cytochrome b_{556} which remained associated upon immunoprecipitation (Crowe and Owen, 1983). In neither of these cases has the cytochrome been shown to be a subunit of the enzyme complex. With the beef heart enzyme, it seems likely that one of the small subunits is the apocytochrome and would confer upon the enzyme the ability to interact with quinone (Ackrell *et al.*, 1980).

It seems striking that fumarate reductase is the only enzyme of this group where a cytochrome has not been implicated as a subunit. This may be a reflection of key functional differences between succinate dehydrogenases and fumarate reductase.

THREE SUBUNIT OXIDOREDUCTASES

As seen in Table VII.2, the succinate dehydrogenase of *B. subtilis* and the fumarate reductase of *W. succinogenes* are composed of only three subunits each. The largest subunits of 65,000 and 79,000 daltons respectively, contain covalently linked FAD (Hederstedt and Rutberg, 1981; Unden *et al.*, 1980). Unlike other fumarate succinate oxidoreductases, the flavoprotein subunit of *W. succinogenes* is capable of fumarate reduction in the absence of the iron sulfur subunit (Unden and Kröger, 1981).

The *B. subtilis* enzyme showed evidence for the presence of three iron sulfur centres. The EPR characteristics were very similar to those of the bovine enzyme. The potentials for the clusters were not determined, although the spin relaxation enhancement caused by centre 2 was titrated at -300mV (Hederstedt *et al.*, 1985). The location of the iron sulfur centres is still uncertain, but a fragment of at least 15,000 daltons of the B subunit was required for assembly.

Only the flavoprotein subunit of *W. succinogenes* fumarate reductase has been shown to contain a [4Fe-4S] cluster

(Albracht *et al.*, 1981). The 31,000 dalton subunit contains a [2Fe-2S] cluster. The presence of a third cluster analogous to centre 2 has not reported.

A b-type cytochrome is present in each of the enzymes in this class. Cytochrome b_{558} associated with the *B. subtilis* enzyme, is required for membrane attachment (Hederstedt, 1980).

The cytochrome subunit (25,000 daltons) of the *W. succinogenes* enzyme is present at two copies per complex (Unden *et al.*, 1980). It is also responsible for membrane binding and for interacting with the electron transport chain (Unden and Kröger, 1981).

The significance of the replacement of two small anchor subunits present in the four subunit oxidoreductases by a single subunit in the three subunit oxidoreductases, remains to be elucidated.

B. DISCUSSION

The studies reported in this thesis were directed towards an understanding of the structural and functional properties of the fumarate reductase of anaerobically grown *E. coli*. Much of the early work was stimulated by the completion of the nucleotide sequence of the *frd* operon. Some of the work refined our concepts of the organization of fumarate reductase in the plasma membrane. Initially, our focus was on the demonstration of the existence and importance of the newly discovered polypeptides coded for in the *frd* operon. Our concept of a dimeric fumarate reductase was soon expanded to include four subunits when the C and D subunits were found to be co-expressed, co-extracted and co-purified with the A and B subunits.

The hydrophobic nature of the new subunits pointed to their role in membrane attachment. Both molecular biological and biochemical approaches contributed to our present understanding of this function. The C and D subunits also served to modify catalytic activity of the dimer, transforming it to a tetrameric form independent of anions and more stable to extremes of pH or temperature. A number of mysteries were resolved by the discovery of the membrane anchors.

In Chapter IV, we further refined our model for the enzyme by including secondary structure prediction information for the anchor subunits. The models remain to be rigorously tested. Classical approaches addressing the question of topology have been inconclusive.

The nature of the interactions which bind the catalytic dimer to the anchor subunits remains to be addressed. It seems enigmatic that the C and D subunits are basic proteins while the catalytic dimer is modulated by anions. Refinement of the reconstitution procedure used in Chapter III, might prove useful in identifying key residues involved in these interactions. We do not yet know which subunits participate in these interactions. Are both the C and D subunits required for anchoring? Our concept of the structure of fumarate reductase has changed considerably, but many features need clarification.

A second theme present in this work, is the characterization of the effects of overproduction of a membrane enzyme. Soon after we achieved 20 to 30 fold amplification of fumarate reductase, we detected a novel tubular organelle in the cell. The tubules are a lipid protein structure which apparently contains fumarate reductase as the only protein arranged in a helical array. Our initial interest was phenomenological, but we soon became concerned with the origin, assembly and function of the organelles. How and why the cell responds to the overproduction of fumarate reductase by increasing phospholipid synthesis, remains to be elucidated.

Surprisingly, amplification of the reductase does not seem to hamper the bacterium's growth potential as measured by growth rates or yields. The organism maintained a normal lipid to protein ratio and apparently did not displace other membrane bound activities to the cytoplasm to accomodate the reductase. The ability to sequester the excess enzyme as a regular array in the tubules, implies quaternary interactions between holoenzyme complexes. These interactions may prove useful in obtaining high resolution structural data from two or three dimensional crystals.

The various aspects of material presented here, summarize our attempts to gain an understanding of the function of fumarate reductase as a member of the electron transport chain. The material in Chapter VI significantly contributes to this understanding. We have expanded the roles of the anchor subunits to include participation in electron transport. This latest function may explain the need for two different membrane subunits rather than two copies of a single one. Elucidation of the pathway of reducing equivalents within the holoenzyme should remain a priority. These studies would eventually clarify the nature of the interactions between *frd*C and the flavin and iron sulfur cofactors present in the catalytic subunits.

Mutant enzyme from GS19 lacked the ability to oxidize quinone. Prior to this discovery, came the realization that normal holoenzyme could perform this function. This forced us to question the location and role of the b-type cytochrome associated with the electron transport chain. Menaquinone was now implicated as the immediate donor of electrons to the reductase.

Much remains to be explained about the electron transport chain to fumarate. Of central importance is the cytochrome which has proved refractory to isolation and characterization. Its purification will be essential to achieving the reconstitution of a coupled electron transport chain from glycerol-3-phosphate to fumarate. Prior to leaving the lab, I generated a bank of mutants selected so as to be limited to cytochrome or reductase mutants. Perhaps the identification of a cytochrome mutant will prove a key advancement in our understanding of this system.

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